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Evaluation Of Immunogenicity Of Transgenic Chloroplast Derived Protect

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EVALUATION OF IMMUNOGENICITY OF TRANSGENIC CHLOROPLAST DERIVED PROTECTIVE ANTIGEN OF *BACILLUS ANTHRACIS*

by

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ABSTRACT

Anthrax, a fatal bacterial infection is caused by *Bacillus anthracis,* a gram-positive, spore forming, capsulated, rod shaped organism. Centers for Disease Control (CDC) lists anthrax as Category A biological agent due to its severity of impact on human health, high mortality rate, acuteness of the disease and potential for delivery as a biological weapon. The currently available human vaccine in the United States (AVA anthrax vaccine adsorbed) is prepared from Alum adsorbed formalin treated supernatant culture of toxigenic, non-encapsulated strain of *Bacillus anthracis* with the principle component being protective antigen (PA83). Evaluation of anthrax vaccine given to nearly 400,000 US military personnel by Vaccine Adverse Event Reporting System (VAERS) showed adverse effects such as flu-like symptoms, local pain, large degree of inflammation, edema, malaise, rash, arthralgia, and headache following vaccination. All the adverse reactions are attributed to the composition of vaccine components. These vaccine preparations contain trace contaminants of lethal and edema factors that contribute to the adverse side effects. Also, the current method of vaccine manufacture has limited production capacity.

The production of PA83, in plants through chloroplast genetic engineering might eliminate the concerns of adverse side effects and the levels of expression would ensure the availability of vaccine for the human population in an environmentally friendly approach. The primary concern is whether the PA83 purified from transgenic chloroplasts is as immunogenic as the PA83 in the AVA. For this, PA83 has been expressed in transgenic chloroplasts of *Nicotiana tabacum var. petit Havana*, by inserting the *pag*

(2205 bp) with the N-terminal 6X histidine tag, into the chloroplast genome by homologous recombination. Chloroplast integration of the *pag* was confirmed by PCR and Southern analysis. The PA83 protein was detected in transgenic chloroplasts by immunoblot analysis using anti-PA83 antibodies. Maximum expression levels of PA83 (14.17% TSP) were observed in mature leaves upon continuous illumination, due to the presence psbA 5'UTR, a light and developmentally regulated translation enhancer sequence. The PA83 has been purified by affinity chromatography using Ni resin columns. Chloroplast derived PA83 was functional *in vitro* and was able to lyse the mouse macrophages when combined with the lethal factor. The *in vitro* assays showed that the crude extracts contained up to 20ug/ml of functional PA83.The immunization studies of PA83 on Balb/c mice, revealed highly immunogenic IgG titers. Subcutaneous immunization with purified chloroplast derived PA83 with adjuvant yielded IgG titers up to 1:320,000, similar to that of the group immunized with PA83 derived from *Bacillus anthracis.* Immunization of groups with PA83 combined with alhydrogel adjuvant showed four - eight times higher immune response than the groups without adjuvant. The higher expression levels of PA83 in transgenic chloroplasts might ensure the availability of anthrax vaccine to the general public and the high immune response observed in the mouse model would enable the replacement of the current AVA with a cleaner and safer vaccine.

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LIST OF ACRONYMS/ABBREVIATIONS

PA-Protective antigen

Pag- Protective antigen gene.

INTRODUCTION

Background and history of Anthrax

Anthrax, a fatal bacterial infection is caused by *Bacillus anthracis*, a Gram-positive spore-forming organism. It is a zoonotic disease transmitted from animals to humans. CDC lists anthrax as Category A biological agent due to its severity of impact on human health, high mortality rate, acuteness of disease and potential for delivery as a biological weapon. The disease is acquired when the spores enter the body either through the skin or inhalation or ingestion (Dixon *et.al*.,1999). Animals acquire infection while grazing on pastures contaminated with Bacillus spores. Humans acquire infection either when they come in contact with contaminated animals or affected humans. There are different forms of anthrax based on the route of infection. Cutaneous is anthrax the most common form, results from contact with contaminated animals or animal products. The cutaneous form can be treated with proper use of medication and is not fatal unless it becomes systemic. Inhalational anthrax, the fatal form results upon inhalation of spores. The mortality is almost 100% and death occurs usually within few days after appearance of symptoms. In the Inhalational form, systemic infection occurs with bacteremia and toxemia leading to shock and death. Gastrointestinal anthrax, often results due to consumption of contaminated food /feed with spores. The mortality is variable.

Historically anthrax has been described in the fifth Egyptian plague (1500B.C.). Virgil, an ancient Roman poet recorded the description of anthrax in 25 B.C. This disease was popularly known as “Black Bane” in the middle ages. In 1870s Robert Koch

extensively studied *Bacillus anthracis* and demonstrated for the first time the bacterial origin of a specific disease (Pile *et al*., 1998). It served as the prototype for Koch's postulates regarding the causation of infectious disease. The first vaccine containing attenuated live organisms was Louis Pasteur's veterinary anthrax vaccine. In the 1900s, human inhalation anthrax occurred sporadically in the United States among textile and tanning workers. Eighteen cases of inhalational anthrax were reported in the United States from 1900 to 1976 (Inglesby *et.al*., 2002). An outbreak of inhalation anthrax occurred in Sverdlovsk near a Soviet military microbiology facility in 1979. This epidemic represented the largest documented outbreak of human inhalation anthrax in history (Sternbach, 2003). In October and November 2001, as a result of intentional release of the organism in the United States via US postal mail, 11 persons contracted pulmonary anthrax, and seven persons contacted the cutaneous form of the infection. Five of those with pulmonary anthrax died. (Inglesby *et.al*., 2002).

The deliberate spread of anthrax spores through U.S.mail resulting in 5 deaths from inhalational anthrax has indicated the threat of anthrax being used as a biological weapon. The research on anthrax as a biological weapon began more than 80 years ago. In 1915, attempts were made by Germans to develop biological weapon using *Bacillus anthracis* spores. In 1937, Japan used *B. anthracis* as a biowarfare weapon against the Chinese. Other countries, specifically the USA, the UK, and the Soviet Union, also started developing *B. anthracis* as a weapon before and during World War II. More recently, Iraq has been implicated as having an active biological warfare program, utilizing a number of agents, including *B. anthracis* (Schmid and Kaufmann., 2002).*B. anthracis* has many biological, technical and virulence characteristics that make it

attractive as a bioweapon. *B. anthracis* is relatively easily obtained from a variety of sources. Once obtained, it is relatively easy to grow and process. The weaponized agent can be easily stored after production. It can be prepared with minimal technology, and, as the recent outrage has shown, it is easily delivered and engenders great public panic. When used as a weapon of mass destruction, the agent is dispersed in particles less than 5μm in diameter, a size that allows penetration into the pulmonary alveoli. Aerosol dispersion can expose the greatest number of people over time (Oncu *et.al*., 2003). The World Health Organization estimated that 50 kg of weapon-grade anthrax spores released by an aircraft over an urban population of 5 million would result in 250,000 cases of predominantly inhalational anthrax. The economic impact was estimated at $26.2 billion per 100,000 persons exposed (Oncu *et.al*., 2003). This large scale mass destruction creates the need for large scale prophylactic measures involving production and stockpiling of vaccines and drugs that counteract the infection.

Bacillus anthracis is gram positive, anaerobic, capsulated, sporulating, rod shaped bacteria. The virulent strains of *B. antharcis* carry plasmids pX01 that carries genes encoding the toxins and pX02 that encodes for the poly-D-glutamic acid capsule (Brossier and Mock., 2001). PX01 carries the genes *pag*A, *lef* and *cya* that encode for protective antigen, lethal factor (LF) and edema factor (EF) respectively. The term protective antigen is derived because of its ability to elicit a protective immune response against anthrax. None of these proteins are toxins individually. However, the protective antigen in combination with edema factor, known as edema toxin causes edema. Similarly the protective antigen in combination with lethal factor forms lethal toxin (Welkos *et.al*., 1998, Mogridge *et.al*., 2001). The ORF of the protective antigen gene

(*pag*) is 2319 bp long of which 2205 bp encode 735 amino acids of the mature protein (PA83). This region is preceded by 29 codons, which encode signal peptide (Welkos *et.al.*, 1988.) The monomer is organized mainly into anti-parallel beta-sheets and has four domains. Domain 1(residues 1-249) is an amino-terminal domain containing two calcium ions and the cleavage site for activating proteases; Domain 2 (residues 250-487) a heptamerization domain, contains a large flexible loop implicated in membrane insertion; Domain 3 (residues 488-594) is a small domain which has the hydrophobic patch that is thought to be implicated in protein protein interaction; and Domain 4 (Residues 595-735) is a carboxy-terminal receptor-binding domain. The Protective antigen binds to the anthrax toxin receptor (ATR), a membrane protein. (Bradley *et.al*., 2001). Once the carboxy-terminal of PA binds to the ATR, it is cleaved into two fragments by furin family of cell-associated proteases. Removal of a 20K amino-terminal fragment from domain 1 allows the PA63 fragment bound to the anthrax toxin receptor (ATR) and exposes a hydrophobic surface for binding the toxic enzymes, EF and LF (Petosa *et al*., 1997, Brosssier and mock, 2001, Mogridge *et al*., 2002). This interaction is via amino terminal domains of EF and LF (Mogridge *et al*., 2002). Each of the toxic enzymes exhibits competitive binding for PA (Gupta *et al*., 1999.) . The PA63 forms an assembly of the heptamer, a ring-shaped structure with a negatively charged lumen, and exposes the hydrophobic surface (Petosa *et al*., 1997, Brosssier and Mock, 2001, Mogridge *et al*., 2002). After the toxin components bind to the hydrophobic surface of PA63, the hepatamer gets internalized by receptor mediated endocytosis into the lumen of endosomes where PA63 is believed to interact with the endosomal membrane, forming a channel to facilitate the translocation of EF and LF across the lipid bilayer into the

cytosol (Jill *et al*., 1994, Milne *et al*., 1994). Proteolytic cleavage of PA83 to PA63 and the low acidic pH in the endosome is required for the membrane insertion and channel formation (Milne *et al*., 1994). LF is a highly specific, four-domain zinc metalloprotease that cleaves members of the MAPKK family within their N-termini. This cleavage initiates a poorly characterized chain of events leading to host death (Inglesby, 1999). A rapid and selective apoptosis of lipopolysaccharide-activated macrophages has been correlated to the LF-induced cleavage of the N-terminal portion of MAPKKs with activation of the p38 mitogen-activated protein kinase (MAPK) (Ascenzi *et al*., 2002). By inducing apoptosis of activated macrophages, LT could prevent the release of chemokines and cytokines that alert the immune system to the presence of infecting *B. anthracis*, thereby facilitating the systemic spread of the infection. The final stages of anthrax have all the characteristics of a septic shock triggered by an extensive release of vasoactive mediators of inflammation. Perhaps LF also cleaves other intracellular substrates or it exhibits additional functions in host cells, which could account for these pathophysiological observations (Chaudry *et al*., 2002). EF is a calcium- and CaM-dependent adenylyl cyclase that takes ATP, separates two phosphates and forms cAMP. EF saturates the cell with cAMP, disturbing water homeostasis and destroying the delicate balance of intracellular signaling pathways. Moreover, EF impairs neutrophil functions and is believed to be responsible for the edema found in cutaneous anthrax (Acenzi *et al*., 2002).

Protective antigen is a primary immunogen and is a component of human vaccines in UK and USA. The currently available human vaccines in the United States and Europe are prepared from Alum adsorbed formalin treated supernatant culture of

toxigenic, non-encapsulated strain of *Bacillus anthracis*. Bioport Corp, Lansing, Michigan, produces the anthrax vaccine in USA. The anthrax vaccine adsorbed (AVA) is licensed in 1970 to be given to all US military active and reserve duty personnel (Inglesby *et al*., 1999). The introduction of the adsorbed anthrax vaccine was followed by a substantial decrease in cases of anthrax among workers in the wool and leather industries, and the disease has become rare in the United Kingdom (Hambleton *et al*., 1984). Data from a 1950s trial of wool-sorters immunized with a vaccine similar to AVA, coupled with long experience with AVA and the United Kingdom vaccine have shown that a critical level of serum antibodies to the *B. anthracis* PA confers immunity to anthrax (Leppla *et al*., 2002, Pittman *et al*., 2000). Although PA in AVA evokes the protective immune response, AVA has limitations that justify the widespread interest in developing improved vaccines consisting solely of well-characterized components, this vaccine contains other cellular elements that contribute to the relatively high rate of local and systemic reactions. To assess the safety of a licensed anthrax vaccine given to nearly 400,000 US military personnel, reports of adverse events submitted to the Vaccine Adverse Event Reporting System (VAERS) were reviewed and evaluated medically. The Anthrax Vaccine Expert Committee (AVEC), reviewed 602 VAERS reports to assess the causal relationship between vaccination and the reported adverse effects and sought to identify unexpected patterns in the occurrence of medically important events. Nearly half the reports noted a local injection-site, with more than one-third of these involving a moderate to large degree of inflammation. Six events qualified as serious adverse effects, and all were judged to be certain consequences of vaccination. Three-quarters of the reports cited a systemic adverse effects (most common: flu-like symptoms, malaise, rash,

arthralgia, headache). (Sever *et al*., 2002). Geier and Geier, 2002 have conducted an analysis to evaluate anthrax vaccine (AVA) and joint related adverse reactions based upon analysis of the VAERS database. The analysis shows a very large and statistically significant increase in joint symptoms following vaccination with AVA when compared to our control population consisting of adverse joint reactions reported following vaccination with hepatitis A vaccine and Td vaccine (Geier and Geier, 2002). All these adverse reactions are attributed to the substandard levels of vaccine production and the composition of vaccine components. The anthrax vaccine is produced by the Health Protection Agency (HPA, Porton Down, UK, Product Licence 1511/0058), and by Bioport Inc. (Lansing, Michigan, US Licence 99). The British anthrax vaccine is produced from supernatant of a static culture of the Sterne strain, a non-encapsulated toxigenic variant of *B. anthracis*, which is filtered through a 0.2 μm filter and adsorbed to aluminium salts after precipitation with alum (Hambelton *et al*., 1984, Turnbill, 1991, Whiting *et al*., 2004). The precipitate contains PA, and it is claimed that the filter selectively retains some of the enzymatic components of the anthrax toxin, EF and LF (Hambelton *et al*., 1984). The American anthrax vaccine is made from a cell-free filtrate of a fermentor culture of nonencapsulated toxigenic strain V770-NP1-R. Aluminium hydroxide is added to precipitate antigens from the cell free supernatant. The precipitate contains predominantly PA, small quantities of LF and trace amounts of EF (Ivins *et al* .,1998, Whiting *et al*., 2004). Serological studies of vaccinated humans and vaccinated guinea pigs suggested that the British vaccine contains more EF and LF and less PA than the American vaccine (Turnbill, 1991). Whiting *et al* recently performed two-dimensional gel electrophoresis to define the protein components of the current UK

anthrax vaccine. It was demonstrated that the major antigen present in the vaccine is PA. The 83 and 63 kDa species are dominant but there are numerous lower molecular weight fragments resulting from proteolytic cleavage. Besides PA, the presence of the toxin components, edema factor and lethal factor, and S-layer proteins, EA1 was also demonstrated. Mass spectrometry has also revealed the presence of several bacterial cell-derived proteins present in the vaccine, including PA, enolase, fructose-bisphosphate aldolase, nucleoside diphosphate kinase and a 60 kDa heat shock protein (Whiting *et al*., 2004). These vaccine preparations contains traces of lethal and edema factors that contribute to the side effects such as local pain and edema (Leppla, 2002). Also, the current vaccine supplies are limited. It will take years before increased production efforts can make available sufficient quantities of vaccine for civilian use. (Inglesby *et al*., 1999). Therefore, an effective expression system that can provide a clean and safe vaccine is required.

Chloroplast genetic engineering

The concept of chloroplast genetic engineering was first conceived in the mid 80s with the introduction of isolated intact chloroplasts into protoplasts (Daniell and Dhingra, 2002). Later focus was laid on the development of chloroplast systems capable of efficient, prolonged protein synthesis and the expression of foreign genes (Daniell and McFadden, 1987). Transformation using biolistics has made it feasible to transform plastids without the need to isolate them (Klein *et al.,* 1987). In 1998, the first successful chloroplast genome complementation was reported for the unicellular green alga having

single chloroplast, *Chlamydomonas reinhardtii* (Boynton *et al*., 1988). For this, the photosynthetically incompetent mutants that lacked *atpB* gene were used. These mutants lacked chloroplast ATP synthase activity. The wild-type *atpB* gene was introduced into the cells using tungsten microprojectiles coated with atp gene (Klein *et al*., 1987). The single large chloroplast provided an ideal target for DNA delivery. The wild-type *atpB* gene introduced into cells was able to correct the deletion mutant phenotype which was demonstrated by the restoration of photoautotrophic growth upon selection in the light. This proved that the wild-type gene carried by the vector was successfully integrated into the *C. reinhardtii* chloroplast genome via homologous recombination, replacing the deleted *atpB* gene. Later, using the *uidA* gene, it was then demonstrated that the foreign DNA flanked by chloroplast DNA sequences is incorporated and stably maintained in the *C. reinhardtii* chloroplast genome; although the introduced *uid*A gene was transcribed, translated product could not be detected (Blowers *et al*., 1989). After the successful transformation of the unicellular algae *C. reinhardtii* through chloroplast genome, efforts were made to transform the higher plants through chloroplast genome. Initially in higher plants, foreign genes were introduced and expressed only in isolated but intact plastids (Daniell and McFadden, 1987). The first transient expression of a foreign gene in plastids of cultured tobacco cells used autonomously replicating chloroplast vectors (Daniell *et al*., 1990). This work was repeated in wheat leaves, calli and somatic embryos (Daniell *et al*., 1991). Simultaneously, the *C. reinhardtii* chloroplast genome was transformed with the *aad*A gene conferring spectinomycin or streptomycin resistance (Goldschmidt-Clermont, 1991); This became a major breakthrough because the majority of higher plants genetically transformed via the chloroplast genome now use aadA gene as

selectable marker. Stable integration of the *aad*A gene into the tobacco chloroplast genome was then demonstrated (Svab and Maliga, 1993). Initially, when the transgenes were introduced via the chloroplast genome, it was believed that foreign genes could be inserted only into transcriptionally silent spacer regions within the chloroplast genome (Zoubenko *et al*, 1994). However, (Daniell *et al*.1998) advanced forwarded the concept of inserting transgenes into functional operons and transcriptionally active spacer regions. This approach facilitated the insertion of multiple genes under the control of a single promoter, enabling the coordinated expression of transgenes (Daniell and Dhingra, 2002). Chloroplast genetic engineering technology is currently applied to other useful crops such as potato, tomato, carrot, cotton and soybean by transforming the plastids (Sidorov *et al*., 1999; Ruf *et al*., 2001; Kumar *et al*., 2004a, Kumar *et al*., 2004b, Dufourmantel *et al*., 2004).

Tobacco, a non-food/ feed crop is proven to be ideal for transformation because of its ease for genetic manipulation. Tobacco is an excellent biomass producer (in excess of 40 tons fresh leaf weight/acre based on multiple mowings per season) and a prolific seed producer (up to one million seeds produced per plant), which is ideal for the large-scale commercial production. It has been extensively used for the large-scale production of therapeutic proteins production. It has been estimated that the cost of production of recombinant proteins in tobacco leaves will be 50-fold lower than that of *Escherichia coli* fermentation systems (Kusnadi, 1997). Using the chloroplast transformation, tobacco has been used for hyper-expression of vaccine antigens and production of valuable therapeutic proteins like human elastin-derived polymers for various biomedical applications (Guda *et al*., 2000), vaccines antigens for cholera (Daniell *et al.,* 2001a),

monoclonal antibody. Guy's 13, a monoclonal antibody against *Streptococcus mutans* to protect against dental carries, have also been expressed in the chloroplast (Daniell *et al*., 2001b). Human therapeutic proteins, including human serum albumin (Fernandez *et al*; 2003), magainin, a broad spectrum topical agent, systemic antibiotic, wound healing stimulant and a potential anticancer agent (Degray *et al*; 2001), interferon (Daniell *et al*; 2004a) and insulin- like growth factor (Daniell, 2004) have been expressed. Several other laboratories have expressed other therapeutic proteins, including human somatotropin (Staub *et al*; 2000) and interferon-GUS fusion proteins (Leelavathi and Reddy, 2003) in transgenic chloroplasts.

The chloroplast transformation technology has been used to introduce the various agronomic traits. Insect resistance has been achieved by expressing insecticidal proteins from *Bacillus thuringiensis* (Bt); the Cry2Aa2 protein had accumulated up to 46.1% tsp and this is by far the highest expressed foreign protein in transgenic plants to date. (DeCosa *et al*., 2001). Similarly herbicide resistance against Glyphosate, a broad-spectrum herbicide that non-selectively kills the weeds by inhibiting the 5-enolpyruvylshikimate- 3-phosphate synthase (EPSPS), a nuclear-encoded chloroplast-localized enzyme in the shikimic acid pathway of plants and microorganisms that is required for the biosynthesis of aromatic amino acids. The plastid that was engineered with *EPSPS* gene in tobacco plants developed resistance to glyphosate over the wild type plants (Daniell *et al*., 1998). The antimicrobial peptide MSI-99, an analog of magainin has been expressed in the chloroplast genome of transgenic tobacco upto 21.5% TSP (De gray *et al*., 2001). MSI-99, offers protection against prokaryotic organisms due to its high specificity for negatively charged phospholipids found mostly in bacteria. Extracts from

MSI-99 transformed plants inhibited growth of *Pseudomonas aeruginosa*, a multi-drug resistant bacteria, which acts as an opportunistic pathogen in plants, animals, and humans. It is also biologically active against *Pseudomonas syringae*, a major plant pathogen. (DeGray *et al*. 2001, Devine and Daniell, 2004, Daniell *et al*., 2004a). The yeast trehalose phosphate synthase (TSP1) gene expressed in the chloroplast showed a high degree of drought tolerance (Lee *et al*., 2003). The Daniell lab recently engineered the carrot plastid genome with the BADH gene, which conferred tolerance to salt (Kumar *et al.*, 2004a).

Vaccine antigens Expressed Via the Chloroplast Genome

Expressing vaccine antigens via the chloroplast genome has proven to be advantageous: subunit vaccines which do not express active toxins are safe and do not multiply; bacterial genes have high AT content allowing for high expression in the chloroplast; and oral delivery of vaccines yields high mucosal IgA titers along with high systemic IgG titers, enabling the immune system to fight against germs at portals of entry. Vaccines that have already been expressed in the chloroplast include the C terminus of *Clostridium tetani* (TetC) (Maliga P, 2003), the Cholera toxin B-subunit (CTB), which does not contain the toxic component that is in CTA (Daniell *et al*. 2001a), the 2L21 peptide from the Canine Parvovirus (CPV) (Molina *et al*., 2004) and the F1~V fusion antigen for plague (Singleton *et al*., 2003).CTB was expressed at 4 to 31% of Total Soluble Protein (TSP) and was effective in the G_{M1}-ganglioside binding assay which indicates proper folding and formation of disulfide bonds to form pentamers (Daniell *et*

al. 2001a, Molina *et al*., 2004). The C terminus of *Clostridium tetani* (TetC) was expressed at 25% TSP for AT rich and 10% TSP for GC rich sequences which shows that chloroplasts favor prokaryotic-AT rich sequences. TetC when administered intranasally, produced both IgG and IgA and was immunoprotective against the toxin (Tregoning *et al*., 2003). The 2L21 peptide from the Canine Parvovirus (CPV) fused to GFP expressed 22% TSP and CPV fused to CTB 31% TSP (Molina *et al*., 2004)**.** When mice were immunized intraperitoneally with the leaf extracts from CTB-2L21, the developed anti-2L21 antibodies able to recognize VP2 protein from CPV. To date, only two vaccine candidates derived from chloroplast genetic engineering have been tested for immunogenecity in mice and only one vaccine candidate has been tested for immunoprotective property. High expression is not only economically important, but for oral vaccines it is essential for the immune response.

Bioencapsulation for the oral delivery of vaccine antigens and protection of immunogens in the Gut

Bioencapsulation of pharmaceutical proteins within plant cells offers protection against digestion in the stomach yet allows for successful delivery (Walmsley and Arntzen, 2000; Yu and Langridge, 2001). In human clinical trials performed with plant derived vaccines, plant cells have proven sufficient for vaccinogen protection against digestion, and the vaccinogen has induced systemic and mucosal immune responses without the aid of adjuvants (Tacket *et al*., 1998, Kapusta *et al*., 1999, Tacket *et al*., 2000,Walmsley and Arntzen, 2000,Tacket *et al*., 2003 Tacket *et al*., 2004). Heat-labile

enterotoxin B-subunit (LTB) from *E. coli* was expressed by nuclear transformation in tobacco (<0.01% Total soluble protein) and potato (0.19% TSP). The LTB expressed in potato was found to be immunoprotective when administered orally. Inspite of lower expression in tobacco these antigens were immunogenic (Haq *et al*., 1995, Mason *et al*., 1996, Tacket *et al*., 1998). The capsid protein of the Norwalk virus expressed in potato and tomato was immunogenic when administered orally (Mason *et al*., 1996, and Richter *et al*., 2000, Tacket *et al*., 2000). The envelope surface protein of hepatitis B virus was expressed by nuclear transformation in tobacco, potato and lupin. They all had less than 0.01% fresh weight expression but were still immunogenic (not protective) when administered orally (Richer *et al*., 2000, Kapusta *et al*., 1999). This again brings us to the need to ensure high expression of therapeutic proteins in plants. IFN-alpha given orally has biologically activity in humans and other animals (Bocci 1999). Plant derived edible vaccines have also proven in commercial animal and native animal trials (Castanon *et al*., 2000, Tuboly *et al*., 2000). Bioencapsulation is therefore protecting these antigens or therapeutic proteins from digestion so that they remain biologically active. Chloroplast genetic engineering is currently being applied to crops amiable to oral vaccines such as potato, tomato, carrot and soybean (Sidorov *et al*., 1999, Ruf et al., 2001, Kumar *et al*, 2004a, Dufourmantel et al., 2004).

Advantages of Chloroplast Expression Over Expression in *E. coli*

Chloroplast expression system has several advantages over *E. coli* expression system. Production of recombinant proteins in microorganisms is expensive, requires

stringent purification protocols, and scale-up requires building costly fermenters. Vaccine production in plants can circumvent these problems. First, farming of plants is straightforward, fairly inexpensive, and can be scaled-up at low cost in one season. The cost of production of recombinant proteins in tobacco leaves will be 10 to 50 times cheaper than that of *E. coli* fermentation (with 20% expression levels in *E. coli*, Kusnadi *et al*., 1997).

Second, plants provide a heat-stable environment for proteins, and the technology already exists for harvesting, storage, and purification of transgenic plant proteins. In addition, each transgenic plant generated can produce up to a million seeds per plant .

Third, the use of plants to produce vaccines eliminates the threat of other components of *B. anthracis* from entering the vaccine. It also avoids contamination with animal pathogens.

Fourth, chloroplasts are capable of folding proteins and maintaining their natural conformation. Folding, assembly and production of disulfide bridges for CTB has already been demonstrated in chloroplasts (Daniell 2001a). Binding assays proved that chloroplast synthesized CTB binds to the G_{M-1} ganglioside receptor. Many antigens for vaccines that have been expressed in chloroplasts have been proven to be immunogenic (Tregoning *et al*., 2003, Molina *et al*., 2004).

Lastly, the process of purification of a recombinant protein produced in *E. coli* is costly and time consuming. For example, for insulin production, chromatography accounts for 30% of the production cost and 70% of the set-up cost (Petrides *et al*. 1995). A transformed tobacco plant would still need purification. But an edible vaccine such as in the carrot or tomato would eliminate this cost. Expenses for delivery by injection

would also be eliminated. Therefore, expression of PA in the carrot plastids for oral vaccination would be beneficial. The transgenic chloroplast derived PA will have to be tested to prove its functionality invivo. This work would pave the way for the oral delivery vaccine in carrot.

Advantages of Chloroplast Transformation Over Nuclear Transformation

Cross Pollination with pollen from Genetically Modified crops with the wild type crops is a major concern. However, by genetically engineering the chloroplasts, the possibility of cross-pollination with pollen carrying transgenes is eliminated because chloroplasts DNA are maternally inherited. Although pollen from plants was shown to exhibit maternal plastid inheritance through metabolically active plastids, the plastid DNA itself is lost during the process of pollen maturation and hence is not transmitted to the next generation. During fertilization, the paternal chloroplasts from pollen are disintegrated in a synergid cell and only the sperm nucleus enters the egg cell and fuses with the egg to form a zygote. Maternal inheritance thus offers the advantage containment of chloroplast transgenes due to lack of gene flow through pollen (Daniell, 2002, Daniell and Parkinson, 2003). The transgene inheritance thus occurs only via seeds. This environmental eco-friendly feature should eliminate all the concerns of cross contamination with wild type and relative crops.

In order for plant production of proteins to be commercially feasible, expression levels greater than 1 % of the total soluble protein must be achieved in plants (Kunsnadi *et al*., 1997). Nuclear transformation of plants has usually produced lower expression

levels of antigens (Daniell *et al*., 2001a, May *et al*., 1996, Richter *et al*., 2000, Tacket *et al*., 2000, Ramirez *et al*., 2003, Devine and Daniell, 2004, Daniell *et al*., 2004b, Daniell *et al*., 2004c). For example, plant derived recombinant hepatitis B surface antigen was as effective as a commercial recombinant vaccine, but the levels of expression in transgenic tobacco were low (0.0066% of total soluble protein).

For an oral delivery vaccine, it may prove to be extremely important to have high expression levels in order to elicit the immune response. An alternative approach to nuclear transformation is to integrate foreign genes into the chloroplast genome, which is a powerful technique because of the number of copies of chloroplast genomes per cell up to 10,000). This high polyploidy leads to high transcript levels and finally accumulation of abundant translated product resulting in high expression levels upto 47% of (DeCosa *et al*., 2001).

Chloroplast transformation occurs exclusively through site-specific homologous recombination. In contrast, nuclear transformation experiments frequently suffer from gene-silencing mechanisms resulting in unstable and inconsistent gene expression or complete loss of transgenic activity. The nuclear genome has mechanisms that may inactivate genes when regulatory sequences are inserted in a repetitive pattern. This occurs because integration of transgenes into the nuclear genome is random and not via homologous recombination (Daniell and Dhingra, 2002). This random integration of transgenes may allow for insertion of the transgene into a region of the nuclear genome that is not highly transcribed. Due to the random position of the transgene in the nuclear genome, expression levels vary in different transgenic lines. However, neither gene silencing nor position effects have been observed in genetically engineered chloroplasts

(Daniell, and Dhingra, 2002). Chloroplast transgenic lines with accumulation of transcripts 169-fold higher than nuclear transgenic lines have shown no gene silencing (Lee *et al*., 2003, Dhingra *et al*., 2004). Likewise, chloroplast transgenic lines showed no transgene silencing at the translational level regardless of accumulation of foreign protein up to 47% TSP (DeCosa *et al*., 2001). Besides, the chloroplast genetic engineering offers attractive advantages of introducing multigenes in a single transformation step because of its ability to transcribe the operon with multigenes into polycistronic mRNA and translate the polycistronic mRNA. This saves a lot of time to create a transgenic plant expressing multigenes as opposed to the nuclear transformation where several independent transgenic lines have to be created followed by the laborious repetitive breeding. (DeCosa *et al*., 2001, Ruiz *et al*., 2003, Daniell and Dhingra, 2002, Lossl *et al*., 2003).

Advantages of Producing the Anthrax Vaccine in Plant Plastids

1. There are no known human or animal pathogens that affect plants (Streatfield *et al*., 2001), production of PA83/PA63 protein in plants would yield a vaccine free of human pathogens.
2. The PA antigen genes have an A/T content of 67%, which are ideal for chloroplast expression of these proteins. Because chloroplasts are prokaryotic in nature they tend to exhibit higher expression with higher AT content genes.
3. Bacterial proteins have been expressed at extraordinarily high levels in transgenic chloroplasts. This includes AT rich proteins such as Cry2a (67% AT) at 47% Total Soluble Protein (DeCosa *et al*., 2001, CTB (59% AT) at 33% TSP (Daniell *et al*., 2001a),

and Human Serum Albumin (66% AT) up to 11.1% TSP, (Fernandez-San Millan *et al.*, 2003). Disulfide bonds in the above examples were properly formed.

4. The PA antigen is not glycosylated, which is good for our particular system because plastids do not glycosylate proteins.
5. The technology to sow, harvest, store, and transport crops already exists.
6. Plants have up to 100 chloroplasts per cell, each containing about 100 chloroplast genomes. This provides up to 10,000 genomes per cell to efficiently produce the antigens
7. Using the chloroplasts eliminates cross-pollination of the transgene by pollen because chloroplast's DNA is maternally inherited (Daniell, 2002, Daniell and Parkinson, 2003).
8. Transgene integration is specifically targeted to intergenic spacer sites in the chloroplast genome; this eliminates gene silencing and the position effect that is seen in nuclear transformation (Daniell *et al*., 2002; Daniell and Dhingra, 2002).
9. AT rich CTB has been produced in transgenic chloroplasts in our lab and has been shown to have native conformation via the GM_1-ganglioside binding assay. It was produced 410 and 3100-fold higher in transgenic chloroplasts than nuclear transgenic plants at 4.1 and 31% total soluble protein (Daniell *et al*. 2001a, Monila *et al*., 2004).
10. Vaccine antigens have already been expressed in plants and have shown to be protected in the stomach through bioencapsulation. Also, they have been proven immunogenic when administered orally in clinical trials (Tacket *et al*., 1998, kapusta *et al*., 1999, Tacket *et al*., 2000, Castanon *et al*., 2000, Tuboly *et al*., 2000, Walmsley and Arntzen, 2000,Tacket *et al*., 2003 Tacket *et al*., 2004).
11. Using plastid transformation technology, large quantities of vaccine can be produced.

12. Proving that this vaccine is immunogenic when produced in tobacco chloroplasts could justify the expense to engineer oral vaccines by transforming plastids of carrot and tomato. Delivery of plant-derived vaccine to mucosal tissues has been proven to induce both a mucosal and a systemic immune response (Haq *et al*.1995., Mason *et al.*1996, Arakawa *et al*., 1998). Mucosal immunization may give higher protection from aerosolized spores of anthrax by producing s-IgA in the mucosal system.

Rationale and Approach

The objective of the project is to evaluate if chloroplast derived PA83 is immunogenic in the mouse model. If so, evaluate the immunogenicity of the chloroplast expressed PA83 relative to the PA83 derived from *Bacillus anthracis*. To evaluate the imunogencity, experiments were planned to observe the efficacy of purified chloroplast derived PA83 with that of PA83 obtained from *Bacillus anthracis*. In order to obtain the purified chloroplast derived PA83, the purification methods for plant proteins were analyzed to determine the most optimal one. The Affinity chromatography using Ni resin column appeared to be the most feasible with single step purification. For this the transgenic lines had to be obtained that express full length PA83 with his-tag that would aid in easy, efficient and economical method of purification. Observing the chain of events that occur from binding of PA83 to anthrax toxin receptor to the translocation of EF and LF by PA, it appeared that the cleavage of PA83 to the PA63 on the anthrax toxin receptor is the rate-limiting step in the down stream sequence of events. Therefore, we decided to express PA63 with his-tag so that the rate-limiting step can be overcome

eliminating the N-terminal 20kDa protein and observe how functional potency of PA63 would differ from PA63. The strategic approach is to introduce 6X histidine N-terminal to *pag* by performing polymerase chain reaction using primers as described in Materials and Methods section. Protein isolation and purification from plants is a difficult task, due to the complexity of the plant system compared to bacterial or yeast systems (Desai *et al*., 2002). The better yield of the protein PA83 can be obtained by reducing the purification steps. So the use of single step affinity chromatography would lower the loss of PA83 during purification steps. 6X histidine engineered at N-terminal of PA83 will aid in purification process. The PsbA 5'UTR which is a transcription and translation enhancer will be placed upstream of *pag* to enhance translation. (Fernandez-San Millan *et al*., 2003, Dhingra *et al*., 2004). The expressed protein will be purified using affinity chromatography. The purified protein PA83 will be evaluated for immunogencity on the mouse system. The immunogenicity will be evaluated under different parameters. These include the immuogenicity of chloroplast derived PA relative to PA derived from *Bacillus anthracis* when administered subcutaneously; with or without adjuvant, in the purified and crude form. If appropriate results are achieved, the mice will be challenged with the toxin to observe the survival rate. If chloroplast derived PA functions effectively, it can be proposed for the human clinical trials to replace the current PA. This work will encourage the plastid transformation of carrots for the oral delivery of anthrax vaccine.

MATERIALS AND METHODS

General protocols

Preparation of Ultra competent cells (Rubidium Chloride Method)

The ultra competent cells were prepared using rubidium chloride method (http://www.nwfsc.noaa.gov/protocols-/rbcl.html). The ultra competent cells are absolutely necessary for the transformation of bacterial cells with the plasmid. *E. coli* XL1-Blue MRF^{ab} (Stratagene), a disabled non pathogenic, tetracycline resistant strain, has a history of safe laboratory use due to its inability to survive in the environment has been used to prepare the ultra competent cells. The E.coli glycerol stock was streaked on the LB agar plate containing 12.5 µg/ml tetracycline and incubated at 37°C overnight. Single isolated colony was picked and grown in 5 ml of Psi broth containing 12.5 µg/ml tetracycline and incubated at 37°C for 12- 16 hrs in a horizontal shaker at 225 rpm. Approx. 1 ml of the overnight culture was inoculated in 100 ml of Psi broth and was incubated at 37°C for about 2 hours in a shaker at 225 rpm. The optical density (O.D) was checked at 550 nm after two hours and subsequently after each half hour or an hour depending on the O.D value. The culture was continued to grow until it reaches to 0.48 O.D. The culture was kept on ice for 15 minutes. The cells were pelleted by centrifugation at 3000g/5000 rpm for 5 minutes in a sorvall centrifuge. The supernatant was discarded and the pellet was resuspended in 0.4 volume (40 ml) of ice cold TFB-I solution. The cells were re-pelleted at 3000g/ 5000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in (0.04 volume) 4 ml of TFB-II solution

and immediately iced for 15 minutes. This suspension was divided into 100 µl aliquots then quick freezed in dry ice/liquid nitrogen and stored at - 80°C.

Transformation of the Competent *E. coli* XL1-Blue cells

The competent cells of one hundred µl aliquot were taken from -80°C and immediately thawed on ice and transferred to a falcon tube. About one µl (100 ng) plasmid DNA was added to the competent cells and was mixed by gentle tapping. The mixture was incubated on ice for 30 minutes with gentle tapping at after first 15 min. Then the mixture was incubated at 42°C in a water bath for 90-120 seconds then immediately put on ice for two minutes. Approx. 900µl of LB broth was added to cells and were incubated at 37^{o}C for 45 minutes in a horizontal shaker at 225 rpm. The cells were pelleted by spinning at 13,000 rpm for 30 seconds. The nine hundred µl of supernatant was discarded leaving 100µl, followed by resuspending the cells. Two samples, 50µl and 100µl, of the suspension were inoculated on the agar plate with appropriate selection agent and spread with a glass rod. (http://www.nwfsc.noaa.-gov/protocols-/rbcl.html)

Isolation of Plasmid DNA by Alkaline lysis

A Single isolated colony was picked from the LB agar plate and was grown in LB medium for 12-16 hours at 37°C in a shaker at 225rpm. 1.5 ml of the cells were put into

an eppendorf tube and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was resuspended in 150 µl of Solution I (GTE: 50 mM Glucose, 10 mM EDTA, 25 mM Tris, pH- 8) and the mixture was vortexed to resuspend the cells. One µl of RNase (100 mg/ml) was added to each tube and pulse vortexed. One hundred fifty µl of Solution II (0.2N NaOH, 10% SDS) was added to each tube and mixed by gently inverting the tube 6 times. Further, one hundred fifty µl of Solution III (60 ml of 5M Potassium Acetate, 11.5 M glacial acetic acid, 28.5 ml sterile dH_2O) was added and mixed by gentle inverting 6 times. The mixture was centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was transferred into a fresh, eppendorf tube taking care to exclude white debris (which is bacterial chromosomal DNA/SDS/membrane proteins). Then 900 µl of ice cold, ethanol (95%) was added to the supernatant and to precipitate the plasmid DNA. The supernatant was centrifuged at 13,000 rpm at 4°C for 10 minutes. The supernatant was removed and discarded, taking care not to dislodge the plasmid DNA pellet in the bottom. To remove the salts from DNA, 400µl of 70 % chilled ethanol was added and (without mixing) centrifuged for 5 minutes. The ethanol was discarded and the pellet was subsequently dried in the speedvac on medium heat for 3-5 minutes. Dried DNA pellet was resuspended in TE (pH 8.0). Concentration and quality plasmid of DNA was measured by spectrophotometer. DNA was stored at -20°C. The DNA samples were run on a 0.8% agarose gel for 40 minutes at 80 volts to confirm the successful isolation of plasmid (Sambrook *et al.*, 1989).

Construction for pLD-5'UTR-HisPA83 vector for transformation of tobacco Chloroplast

Polymerase Chain Reaction was performed to introduce a 6X Histidine tag and Factor Xa protease cleavage site N-terminal to *pag* using primers forward 5' – ACGGTTTCCCCATATGCACCATCATCATCATCATTCTTCTGGTATAGATGGAA GAGAAGTTAAACAGGAG- 3' and reverse 5'-CTGATTTTGTTAGCTCGAGA-AGCTTTATTAATCAC- 3' (Invitrogen). The plasmid p-Blue script-gene10-*pag* (PA83 gene) was used as the template and PCR amplified product of size approx. 370 bp containing Nde I restriction site at the 5'end and Xho I restriction site at the 3' end was obtained. For a 50 µl reaction volume, the PCR was set as follows: 100ng of plasmid DNA, 5 µl of 10X pfu buffer, 5µl of 2.5 mM dNTP, 1µl of each 10mM primer, 0.5µl pfu DNA polymerase and H_2O to make up the final volume. The PCR amplification was carried for 25 cycles as following: 94°C for 45 sec, 65°C for 45sec, and 72°C for 45sec for the. Cycles were preceded by denaturation for 5 min at 94°C and followed by a final extension for 7 min at 72°C. The 5µl of each PCR products including the contorls were loaded into a 0.8% gel to analyse the PCR product. The PCR product was purified using PCR purification kit (Qiagen). The phosphate groups were added to the PCR fragment to the 5' ends of both strands and cloned into EcoRV digested and dephosphorylated p-Blue script plasmid vector. The ligated plasmid is isolated using miniprep and the PCR product was sequenced using M13 forward (5'-TGACCGGCAGCAAAATG-3') and M13 reverse (5'GGAAACAGCTATGACCATG-3') primers. The PCR product was isolated by digesting the ligated p-Blue script-PCR product with NdeI and XhoI. The PCR fragment

was then cloned into pCR2.1 vector containing the 5'UTR.The pCR 2.1 vector containing the 5'UTR and the PCR product was digested with Kpn I and Hind III (fragment size approx.600bp) and cloned into p-Blue script-*pag*. Finally the p-Blue script containing the 5'UTR, Histidine tag and *pag* was digested with EcoRV and Xba I (fragment size approx.2.5kb) and cloned into tobacco universal vector pLD-ctv.

Construction for pLD-5'UTR-HisPA63 vector for transformation tobacco Chloroplast

Polymerase chain reaction was performed to introduce His tag and Factor Xa protease cleavage site N-terminal to *pag* using primers forward 5' –TAACTTACAACA-TATGCACCATCATCATCATCATTCTTCTGGTATAGATGGAAGAAGTACAAGTG CTGGA- 3' and reverse 5'- TTTCATGAATATTCTCGAGCCATGGTGAAAGAAAAG - 3'.The PCR amplified product of size approx. 190 bp was obtained with Nde I restriction site at the 5'end and Nco I restriction site at the 3' end as shown in fig 2A. The PCR product has the Histag, Factor Xa protease cleavage site, partial *pag* sequence. The PCR product was purified using PCR purification kit (Qiagen). The phosphate groups were added to the PCR product to the 5' ends of both strands and cloned into EcoRV digested and dephosphorylated p-Blue script plasmid vector. The p-Blue script containing PCR product sequenced. The p-Blue script containing the PCR fragment was digested with NdeI and NcoI and cloned into plasmid p-Blue script containing the 5'-UTR-His and *Pag* gene (Full size). The ligated plasmid was digested with EcoRV and Xba I to clone into pLD-CtV (fig 2c).

Bombardment of the pLD-5'UTR-His-PA83 and pLD-5'UTR-His-PA3 vectors

Preparation of gold particles

50mg of gold particles (0.6μm in size) and 1ml of 70% EtOH were placed in a micro centrifuge tube. The mixture was vortexed for 3-5 minutes and then incubated for 15 minutes at room temperature. To pellet the gold particles, a quick centrifugation was done. The supernatant was discarded and 1ml of dH_2O was added to the particles and vortexed. The particles were allowed to settle for 1 minute and then a pulse centrifugation was performed for 3 seconds, the supernatant was discarded. The previous step was repeated three times. The gold particles are stored in 50% glycerol stock at –20°C (Kumar and Daniell, 2004).

Preparation of tobacco tissue culture media

The RMOP media containing MS basal salt mixture (one pack), 30 grams of sucrose, 100mg of myo-inositol, 1ml of benzylaminopurine (BAP: 1mg/ml), 100μl of α-naphtalene acetic acid (NAA: 1mg/ml), 1ml of thiamine hydrochloride (1mg/ml), and water 1 liter) was used to regenerate and select the transgenic plants after bombardment. The pH was adjusted to 5.8 using 1N KOH. Six grams per liter of phytagar was added to the media and autoclaved followed by cooling down and pouring into plates. The MSO media containing 30g sucrose, 1 packet of MS basal salt mixture, and water to 1 liter was

used to generate roots. The pH was calibrated to 5.8 with 1N KOH and 6g per liter of phytagar were added before autoclaving. (Kumar and Daniell, 2004)

Bombardment protocol for tobacco leaves

The bombardment was performed as described previously (Daniell, 1997). For the bombardment, it is most important to maintain the aspectic conditions. For this all the essential equipments were sterilized. The stopping screens, macrocarrier holders, forceps, Watman filter paper, kimpwipes were autoclaved prior to bombardment. The macrocarriers and the rupture discs were sterilized under hood by immersing them in 95% ethanol for 15 minutes followed by drying. Fifty µl of gold particles placed in a micro centrifuge tube followed by 10µl of DNA (1µg/µl). Fifty µl of 2.5M $CaCl_2$, 20µl of 0.1M spermidine-free base were added sequentially to the mixture to ensure proper binding of DNA to the gold particles. Vortexing was done after addition of each component to ensure proper mixing of components and binding of DNA to the gold particles. The mixture was then vortexed for 20 minutes at 4°C. Two hundred µl of absolute ethanol was added to the vortexed mixture at room temperature and followed by a quick spin at 3000 rpm in a microfuge for 30 seconds, supernatant was removed and this wash procedure was repeated four times. Finally the gold particles were resuspended in thirty ul of 100% ethanol. The gold particles with DNA were placed on ice to be used in next two hours. Aseptic tobacco plant *Nicotiana tabacum* variety Petit havana green healthy leaves were cut from a young plant growing in jars containing MSO media and

placed on a Petri dish *(100 x 15)* containing RMOP media with no selection and a Watman filter paper on the top of media. The leaves were placed with the abaxial side upwards. The gene gun (Bio-Rad PDS-1000/He) was sterilized in the inside chamber with 70% ETOH prior to bombardment. The macro carriers were placed on the macrocarriers holders. The gold particles lying on ice were votexed and five µl of gold particles containing the DNA were placed on top of the macro carrier. Vortexing is an important step while placing the gold particles on the macrocarriers. The rupture disc, stopping screens and macrocarrier holders containing the macrocarrier, and the leaf were put in place and secured to proceed with the bombardments. The gene gun and the vacuum pump were turned on, and the helium tank was turned to the open positions and the valve is turned on till the pressure reaches 1350 psi. The vacuum in the gene gun was allowed to build to 28 psi, and was then held briefly and then fired (the fire switch was held until the rupture disk broke at ~1100 psi) After the bombardment, the vacuum was released, and the Petri dish with the leaf was taken out and covered. After the samples were finished they were covered with aluminum foil (to keep them dark) and incubated for 48 hours at room temperature. (Kumar and Daniell, 2004)

Tissue regeneration and selection

The leaves, after two days incubation period, were transferred to 100x25 Petri dish with RMOP media containing 500µg/ml of spectinomycin final concentration as explained by Daniell, 1997. After four to six weeks, the shoots that appeared were cut in 5mm^2 pieces and transferred to fresh RMOP plus spectinomycin for the second round of selection.

Before transferring the shoots to secondary selection, a total DNA extraction and a PCR analysis was performed to confirm the integration into the chloroplast on these putative transgenic lines. Finally, after 4 weeks on secondary selection, the shoots were transferred to a jar that contained MSO media with 500µg/ml spectinomycin (Daniell, 1997), this step is called the third selection. (Kumar and Daniell, 2004)

Plant genomic DNA extraction procedure

The Qiagen DNeasy Kit was used to isolate plant genomic DNA as described in the Qiagen manual. 100mg of tissue sample was taken from the plant using aseptic techniques, placed into a micro centrifuge tube, and grinded by using a micro pestle in 400µl of buffer AP1 and 4µl of RNase A (stock solution 100mg/ml). The mixture was incubated for 10 minutes at 65°C and mixed about 2-3 times during incubation by inverting the tube. 130µl of buffer AP2 were added to the lysate, vortexed and incubated for 5 minutes on ice. Following, a centrifugation was done at maximum speed for 5 minutes and the supernatant was transferred to a Qiashredder spin column (lilac) sitting in a 2ml collection tube. The centrifugation was performed at full speed for 2 minutes. The flow through was transferred to a new tube and 1.5 volumes of buffer AP3/E were added to the lysate and mixed immediately. 650µl of the mixture was applied to a Dneasy mini spin column (clear) and centrifuged for 1 minute at 8000 rpm. The flow through was discarded and the collection tube reused to repeat the previous step with the rest of the sample. The tube with the flow through was discarded and the column was placed in a

supplied 2 ml tube. 500µl of buffer AW were added to the column and centrifuged for 1 minute at 8,000 rpm. The flow through was discarded, and the tube was reused. The Dneasy column was washed once again by using 500µl of AW buffer and by centrifuging it for 2 minutes at maximum speed. The Column was transferred to a clean 1.5ml tube and 100µl of preheated (65°C) buffer AE were directly delivered into the Dneasy membrane. The membrane was incubated for 5 minutes at room temperature and then centrifuged at 8,000 rpm for 1 minute to elute the DNA. The DNA was kept at -20°C.

Confirmation of transgene integration into the chloroplast genome

To confirm the transgene cassette integration into the chloroplast genome, PCR was performed using the primer pairs 3P(5'-AAAACCCGTCCTCEGTTCGGATTGC-3') -3M (5'-CCGCGTTGTTTCATCAAGCCTTACG-3') (Daniell *et al*., 2001a) and to confirm the integration of gene of interest PCR was performed using primer pairs 5P(5'-CTGTAGAAGTCACCATTGTTGTGC-3' and 2M (5'-TGACTGCCCACCTGAGAGC-GGACA-3') (Daniell *et al*., 2001a). Positive control (known transgenic plant DNA sample) and Negative control (Wild type petit havana DNA sample) were used to monitor the PCR reaction. For a 50 ul reaction volume, the PCR was set as follows: 150ng of plant DNA, 5 µl of 10X buffer, 4µl of 2.5 mM dNTP, 1µl of each primer from the stock, 0.5µl Taq DNA polymerase and H_2O to make up the total volume. The amplification was carried during 25 cycles with a program timed in the following way: 94°C for 30 sec, 65°C for 30sec, and 72°C for 30sec for the 3P-3M primer pair and 72°C

for 1min for the 5P-2M primer pair. Cycles were preceded by denaturation for 5 min at 94°C and followed by a final extension for 7 min at 72°C. The 5 ul of each PCR products including the contorls were loaded into a 0.8% agarose gel to confirm the results.

Southern blot analysis of transgenic plants

Restriction Digestion of plant genomic DNA

The total plant DNA was extracted from transgenic T_0 plants as well as from untransformed tobacco plants following the protocol previously explained using Qiagen DNeasy Plant Mini Kit. These steps were performed as described in (Daniell et al., 2004d) The total plant DNA was digested with BglII in a reaction containing: 2ug of DNA, 2µl of 10X buffer (New England Biolabs), 2µL of BglII enzyme (New England Biolabs) and sterile dH_2O to make up the volume upto 20 ul. The reaction was incubated overnight at 37°C. All the samples must contain equal quantity of DNA.

Agarose electrophoresis and DNA transfer

The total 20µl reaction volume was loaded on a 0.7% agarose gel for each of the transgenic plant DNA samples. The digested DNA of wild type plant (*Nicotiana tabacum* var *Petit Havana*) acts as negative control and (unlabeled probe) acts as the positive control. The positive control (unlabeled probe) was prepared by digesting the plasmid

DNA (pBluescript-gene10-*Pag*) with NcoI. The *810 bp* fragment was diluted 20 times and then 1µl of the diluted probe was loaded onto the gel. The gel was run for 2.5 hours at 50 volts. After the run was completed, the DNA was transferred by capillary action to a nylon membrane. The parts of the gel that were not needed were removed, and the upper right corner was cut to help as a guide. The gel was then depurinated by immersing it in 0.25M HCl (depurinaion solution) for 15 minutes (until the color of the dye became yellow). Following, the gel was washed twice in dH_2O for 5 minutes, and then equilibrated in transfer buffer (0.4N NaOH, 1M NaCl, filled to 1 liter with water) for 20 minutes. The four pieces of Whatman paper and the membrane were cut to fit the size of the gel then the upper right corner of the membrane was removed and washed briefly in water. Following, the membrane was equilibrated by immersing it in transfer buffer for 5 minutes. In a glass tray, a stack of two spongy was placed and enough transfer buffer was added to cover the spongy that is in touch with the tray. On top of the spongy two pieces of whatman paper were placed and some transfer buffer was poured to soak the paper and to remove any air bubbles. The gel was placed facing down on the whatman paper and then the nylon membrane was placed with the cut corner touching the cut corner of the gel. Some transfer buffer was added to remove any bubbles. Two Whatman papers, and then a stack of paper towels were placed on top of the membrane. A 500g weight was placed on the paper towels to help the capillary transfer. The set up was left for transfer over night and the next day the membrane was washed on 2X SSC (3M NaCl, 0.3M Na citrate, H_2O, the pH was adjusted with 1N HCl to 7 and water was added to 1L) for 5 minutes. Following, the membrane was allowed to dry on a whatman paper for 5 minutes

and then cross-linked using the Bio-Rad GS Gene Cross Linker at setting C3 (150 m joules). The membrane was wrapped in saran wrap and stored in a dry place until use.

Generation of probes

These steps were performed essentially as previously described (Daniell *et al*., 2004d)The flanking sequence probe was obtained from the plasmid pUC-ct vector that contains the chloroplast flanking sequences for the trnI and trnA. The digestion reaction was setup as follows: 15µl of pUC-ct vector DNA, 2µl of 10X buffer, 1µl of BamHI (NEB), 1µl of BglII and 1µl of dH_2O. The reaction was performed overnight at 37°C and then run in an agarose gel to obtain the desired fragment of 0.8kb. The band was cut out and eluted from the gel as explained before. For the final DNA elution, 50µl of H_2O was used. The gene specific probe was made by cutting out the 0.81kb fragment from pBlue script-gene10-PA in a reaction as follows: 2ug of pBlue-gene10-PA, 2µl of 10X buffer, 1µl of EcoRV (NEB) and 12µl of dH_2O. The reaction was incubated overnight at 37°C. The fragment of interest (0.81kb) was eluted from the agarose gel and checked for the concentration on the spectrophotometer.

Probe labeling

The probes denaturation (45µl of the DNA) was made by incubating the tube at 94°C for 5 minutes, immediately placing the tube on ice for 2-3 minutes and then pulse centrifuging to bring down any droplets. Following, the probe was added to the ready mix (Quant^{tm} G-50 Micro columns, Amersham) and mixed by flicking. 5µl of α^{32}P was added to the tube and the mixture was incubated for 1 hour at 37°C. Once the incubation period was complete, a G50 column was taken and the resin was re-suspended by vortexing, then the cap was loosened about ¼ and bottom plug was removed. Then, placed the column into a micro centrifuge tube with the top cut off and centrifuged for 1 minute at 3,000 rpm. The collection tube was discarded and the column was transferred to a new 1.5ml tube. The DNA probe was added in the center of the resin and spun at 3,000 rpm for 2 minutes and the column was discarded. From the labeled probe 1µl was mixed with 98µl of STE buffer. The mixture was aliquoted into 50µl samples and to each sample 3ml of Opti-Fluor was added. The activity of the radioactive probe was measured in a Beckman LS 5000TD. The two samples plus a blank containing 3ml of Opti-Fluor were placed into the machine holder. The readings were taken by using the auto-read mode of the machine. The amount of probe to be used was determined by calculating the amount of probe needed to yield 2.5 x 10^6 cpm/2ml. The amount of probe was calculated as follows: Reading value (502050) was equal to 0.50 x 10^6 cpm/µl, then multiplied by 50µl of total volume of sample for a total of 35 x 10^6 cpm/µl. Because 5ml of hybridization

solution was used, we needed 6.25 x 10^6 cpm, therefore 6.25 x 10^6 cpm divided by 0.7 x 10^6 cpm is equal to 12.5µl of labeled probe needed.

Prehybridization, hybridization and washing of membrane

The Quick-Hyb solution from Stratagene was mixed and incubated for 5 to 10 minutes at 68°C. Following, the blot was placed into the hybridization bottle with the top facing in toward the solution and 5ml of the preheated pre-hybridization solution was added. The bottle with the membrane was incubated in the Fisher Biotech Hybridization Incubator for 1 hour at 68°C. One hundred µl of salmon sperm DNA was added to the probe and then heated for 5 minutes at 94°C. Following, 1ml of pre-Hyb solution from the bottle containing the membrane was withdrawn and added to the probe solution. Immediately, the content was returned to the bottle. The hybridization bottle was incubated for 1 hour at 68°C. After the hour of incubation, the quick-Hyb solution was discarded into the liquid radioactive waste container. The membrane was washed twice as follows: 50ml of wash solution number 1 (2X SSC and 0.1% SDS) was poured and incubated at room temperature for 15 minutes. The liquid was discarded in the liquid waste container and the step was repeated. A second round of washes was performed twice by pouring 50ml of solution number 2 (0.1X SSC and 0.1% SDS) and incubating it for 15 minutes at 60°C to increase the stringency. The liquid of these washes were discarded into the radioactive liquid container. The radioactive membrane was wrapped around with saran wrap and kept in a radioactive container in the radioactive hood.

Autoradiography

The blots were placed into the film cassette and then taken to the dark room. Using the safe light (red light), the X-ray film was placed into the cassette on top of the blot and the intensifier screen was placed on top of the X-ray film. The cassette with the blot and the film was placed into a black bag to protect against light and then incubated overnight at –80°C. The next day the cassette was taken out from the –80°C, allowed to thaw, and then moved to the dark room where the film was developed.

Characterization of expressed proteins

Extraction of Protein from Transformed *E. coli C*ells

E. coli, XL1-blue cells that have tetracycline resistance gene in their genome, were transformed with pLD-His-PA83 and pLD-His-PA63 were grown in 5 ml of Terrific broth with ampicillin (100 μg/μl) and tetracycline (50 μg/μl) at 37^{o}C for 14-16 hrs. Untransformed *E. coli* cells were also cultured for use as a negative control. The buffers and gels used in this study were made from protocols in SDS-PAGE Buffer System below (Laemmli, 1970). After the boiling step (as described below), samples were immediately loaded into polyacrylamide gels. The 800 μl of cultured cells were centrifuged for 1 minute at 13,000 rpm. Supernatant was discarded from pelleted *E. coli* cells then washed with 1ml of 1x Phosphate-Buffered Saline (PBS: 140mM NaCl,

2.7Mm KCl, 4mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.2). Pellet was resuspended, followed by centrifugation for 1 minute at 13,000 rpm. Supernatant was discarded. 50 µl of 1x PBS was added and mixed well to resuspend the pellet. A mixture containing 425 µl of 2x loading buffer , also called Sample Buffer or SDS Reducing Buffer (1.25 ml of 0.5 M Tris-HCl, pH 6.8, 2.0 ml of 10% (w/v) SDS, 0.2ml of 0.5% (w/v) bromophenol blue, 2.5 ml of glycerol, q.s. with dH_20 to 9.5 ml, and the 25 µl of β-mercaptoethanol was prepared. Equal amounts of sample and the mix of sample loading buffer and β-mercaptoethanol was taken and Boiled for 5 minutes, then immediately loaded samples onto gels. (Sambrook et al., 1989).

Extraction of Protein from Transformed Tobacco Leaves

In order to confirm expression of PA83 and PA63 in tobacco plants extracts were made from Petit Havana plants. These steps were performed as described in (Daniell et al., 2004d). Approx. 100 µg of plant leaf tissue was weighed and ground with a mortar and pestle in liquid nitrogen and put into a microcentrifuge tube. Two hundred µl of extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl-pH8, 0.05% Tween-20, 0.1% SDS, 14 mM BME, 400 mM sucrose, 2 mM PMSF) was added and the samples were mixed for 3 minutes with a micropestle. The samples are centrifuged at 13,000 rpm for 10min to obtain the supernatant containing the soluble proteins. 20 µl of these extracts were mixed with 20 µl of sample loading buffer containing BME was added. Samples were then boiled for 5 minutes and loaded into SDS-PAGE gel.

SDS-PAGE Buffers and Gels

To detect the PA protein expression in the crude extract obtained from *E. coli* cells or crude extract obtained from the transgenic plant tissue extract transformed with pLD-PA83/ pLD-PA63, SDS-PAGE gels were made in duplicate utilizing buffer solutions: (1) Bio-Rad (cat#161-0158), 30% Acrylamide/Bis solution according to the ratio 37:5:1. (2) The resolving buffer (5M Tris-HCl, pH 8.8), was used to make the resolving portion of the gel. (3) The stacking buffer (0.5M Tris-HCl, pH 6.8), was used to make the stacking gel layer, concentrated the samples at top of the resolving gel to improve resolution. (4) 10x Electrode buffer: 30.3g Tris base, 144.0g glycine and 10.0g SDS q.s. to 1000 ml dH_20. and stored at 4°C, (5) 2x loading buffer, also called the Sample buffer and the SDS Reducing Buffer: see previous section. (6) 10% (w/v) Sodium Dodecyl Sulfate (SDS). (7) N,N,N,N'-Tetra-methyl-ethylene diamine (TEMED) from BIO-RAD (cat# 161-0800). (8) 20% Ammonium Persulfate (APS): Dissolved 20 mg of APS into 1ml dH_20 in an micro centrifuge tube and stored at $4^{\circ}C$ for about a month. The 8% resolving gel was made by the following method: Added 4.0 ml of 30% Acrylamide/Bis, 2.5ml of resolving buffer, 3.4 ml dH_20 and 100 µl of 10% SDS to a 50 ml flask. Added 40 µl of 20% APS (#8 above) and then 10 µl of TEMED and used to cast the gel mixture between the two, vertical, glass plates (Mini-Protean 3 Cell gel system, Bio-Rad) leaving about 1.5 cm at the top of glass plates for the stacking gel. The gel is allowed to polymerize for 20 minutes. To make the 4% stacking gel, 1.3 ml of 30% Acrylamide/Bis, 2.5 ml of the stacking buffer, 6.1 ml dH_20 and 100 µl of 10% SDS are

taken together into a flask followed by 40 µl of 20% APS and 10 µl of TEMED. The 4% gel mixture is layered on top of resolving gel, and then the comb is inserted for the formation of wells. After polymerization for about 20 minutes, the comb is removed and the gel was put vertically into PAGE apparatus containing 1x Electrode (running) buffer. 20 µl of protein extract along with the sample loading buffer was loaded along with PA protein standard, and 10 ul protein marker. Gel was ran at 50V until samples stacked onto the top of the resolving gel, then ran gel at 80V for 2-3 hours so that protein marker bands could spread out sufficiently.

Transfer of protein and analysis of Western Blot

The separated proteins were transferred onto a 0.2 µm Trans-Blot nitrocellulose membrane (Bio-Rad) by electroblotting in Mini-Transfer Blot Module at 80V for 45minutes in Transfer buffer (360 ml of 10x Electrode buffer, 360 ml of methanol, 0.18 grams of SDS, 1080 ml distilled H_20). For Western blotting, the proteins were transferred to nitrocellulose membranes and then blocked for one hour in P-T-M (PBS [12 mM Na_2HPO_4, 3.0 mM NaH_2PO_4-H2O, 145 mM NaCl, pH 7.2], 0.5% Tween 20, and 3% Dry Milk) followed by transfer to P-T-M containing anti-PA monoclonal antibody. Membranes were then washed with distilled water and transferred to P-T-M containing goat derived anti-mouse IgG antibody conjugated with Horseradish peroxidase (Sigma, St. Louis, MO). Blots were washed three times with PBST for 15 minutes each time. Then washed with PBS for 10 minutes, followed by addition of Lumiphos WB (Pierce,

Rockford, IL) as a substrate for HRP and incubating at room temp for 5 min for the chemiluminescence. Later the X-ray films were exposed to chemiluminescence and the films were developed in the film processor to visualize the bands.

Comassie Staining of the protein gel

The Comassie staining was performed using the Comassie dye-250 Brilliant blue (Bio-Rad). The concentrated dye was diluted 5 times with distilled water. The protein gel was SDS-PAGE was incubated in the stain for 30 minutes at room temperature followed by destaining over night with destain solution at room temperature. The gel was allowed to dry in Vacuum dryer.

Enzyme Linked Immuno Sorbant assay (ELISA)

The quantification of PA in the plant crude extract was done using the enzyme linked immunosorbent assay (ELISA). 100mg of transgenic leaf samples (young, mature, old) and the wild type leaf samples (young, mature, old) were collected. The leaf samples were collected from plants exposed to regular lighting pattern (16 h light and 8 h dark), 3 day continuous light, and 5 day continuous light. The leaf samples were finely ground in liquid nitrogen, followed by collection of leaf powder into the eppendorf tube. To extract the protein from the plant leaf powder, plant protein extraction buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, pH: 9.6, 0.1% Tween, 5mM PMSF) was used to resuspend

the ground mixture. The Mechanical pestle was used to agitate the leaf tissue so that the all the soluble protein comes out of the chloroplast and cytosol into the buffer. In order to check the protein concentration, the standards, test samples and antibody were diluted in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, pH: 9.6)The standards ranging from 50 to 500 ng/ml were made by diluting purified PA in coating buffer. The standards and protein samples (100 µl) were coated to 96-well polyvinyl chloride microtiter plate (Cellstar) for 1 h at 37 °C followed by 3 washes with PBST and 2 washes with water. Blocking was done with 3% fat-free milk in PBS and 0.1% Tween and incubated for 1h followed by washing. The primary anti-PA antibody (Immuno Chemicals) diluted (1:1000) in PBST containing milk powder was loaded into wells and incubated for 1h followed by washing steps and then again incubated with 100 µl of anti-mouse IgG-HRP conjugated antibody made in goat (American Qualex) (1: 5000) diluted in PBST containing milk powder. The plate was then incubated for 1h at 37 °C. After the incubation the plate was washed thrice with PBST and twice with water. The wells were then loaded with 100 µl of 3,3,5,5-tetramethyl benzidine (TMB from American Qualex) substrate and incubated for 10–15 min at room temperature. The reaction was terminated by additing 50 µl of 2N sulfuric acid per well and the plate was read on a plate reader (Dynex Technologies) at 450 nm. (Modified form of protocol from Ausubel *et al.,* 4th edition).

Bradford assay for protein quantification (Bio-rad manual).

The Bradford assay was used to determine the total protein from the plant extracts prepared as described above. This was used to determine the percent of PA antigen in the total soluble protein extract (or %TSP). An aliquot of plant extract as prepared above was thawed on ice. Extraction buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g NaN_3, 0.1% Tween 20, and 5mM PMSF adjusted to pH 9.6) was used to make Bovine Serum Albumin (BSA) standards ranging from 0.05 to 0.5 µg/µl. Plant extracts were diluted 1:5, 1:10 and 1:20 with extraction buffer. 10 µl of each standard and 10 µl of each plant dilution was added to the wells of a 96 well microtiter plate (Costar) in duplicates. Bradford reagent (Biorad protein assay) was diluted 1:4 with distilled water as specified and 200 µl was added to each well. Absorbance was read. Comparison of the absorbance to known amounts of BSA to that of the samples was used to estimate the amount of total protein.

Macrophage lysis assays

Macrophage lysis assay was performed on the crude leaf extracts. For this approximately 100 mg of leaf tissue was ground finely in liquid nitrogen. Plant proteins were extracted with 200 µl of plant protein extraction buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g NaN_3, 0.1% Tween 20, and 5mM PMSF. The plant tissue along with the buffer were centrifuged for 5 min at 10,000 *g* and the supernatant containing the soluble proteins was placed in a new tube. RAW264.7 macrophage cells were plated in 96-well

plates in 120 µl DMEM medium and grown to 50% confluence. The medium was aspirated and replaced with 100 µl medium containing 250 ng/ml LF. The control plate received medium with no LF to test toxicity of plant material and buffers. In separate 96-well plates, the plant samples were diluted serially two-fold and 40 µl of the dilutions were transferred onto the RAW264.7 cells. so the top row had plant extract at 1:14 dilution. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added after 5–10 h to assess cell death.

Protein Purification

The purification steps were performed as described in Amersham biosciences chromatography manual. The PA protein expressed in the transgenic plant has the His-tag. The HIS affinity chromatography with nickel column 1ml size (Amersham biosciences) was used to purify the protein. The following buffers were prepared to purify the PA protein from the crude extract. To obtain the crude extract, the plant leaves (6grams) were finely ground in liquid nitrogen. The protein was extracted using 15 ml of plant protein extraction buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g NaN_3, 0.1% Tween 20, and 5mM PMSF adjusted to pH 9.6). The mechanical pestle was used to churn the leaf tissue for 15 minutes in order to extract protein from the plant tissue. The extract was transferred to sorvall tube and centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred into a new falcon tube. The supernatant was stored at –80°C before use. The buffer used for purification are as follows: Binding Buffer: 20mM Sodium Phosphate, 0.5M Sodium Chloride, 10 mM Imidazole, pH 7.4., Elution Buffer:

20 mM Sodium Phosphate, 0.5M Sodium Chloride, pH 7.4., Ni-Loading Eluent:100mM Nickel Sulfate solution (Sigma). Purification operations were performed on the Akta prime machine (Amersham biosciences). The Binding buffer is passed into the purification set up through A1 port, Elution buffer through Port B, Nickel sulphate solution through A2 port and water through A3 port. The purification steps are carried out in as following. Washing the column with elution buffer and water, followed by the loading the column with nickel sulfate solution, equilibrating the column with binding buffer, application of sample, followed by washing and gradient elution. The samples were collected in 1000ul volume .The samples corresponding to the peak were aliquoted for further analysis. The flow through was collected to analyze the loss of PA through flow through. The protein aliquots, flow through samples were analyzed for presence / absence of PA using ELISA. The binding buffer, elution buffer were used as negative controls. The fractions containing the purified PA83 were pooled together and dialyzed against phosphate buffer saline (PBS), pH 7.4 using the dialysis cassette 10K MW (Pierce) to replace the Imidazole buffer. The purified PA in PBS was loaded onto the protein concentrator columns (Millipore) and centrifuged at 3000 rpm until to concentrate the protein.

Immunization studies in mice

7 groups of Balb/c mice, 6-7 weeks old (Charles river laboratories) were immunized subcutaneously at 2 sites 100µl per site on day 0. The injection doses were prepared using 5µg of chloroplast derived PA/PA derived from *Bacillus anthracis* and

50µl of alhydrogel adjuvant in phosphate buffer saline so that the final volume is 200ul per dose. This suspension was allowed to mix thoroughly in a shaker at 225rpm at 4°C for 2-3 hrs to allow the PA bind to alhydrogel. The measurement of PA adsorbed to alhydrogel was done according to (McBride *et al*., 1998). Two of the groups were immunized without the adjuvant. The plant protein crude extract has been dialyzed against the PBS to eliminate the protein extraction buffer and replaced it with PBS. The doses were given on day 0 followed by booster doses at the end of 2nd and 4th week. On the 14th day after the last booster blood was drawn from the retro orbital plexus and collected in the microtainer tubes (Fisher Scientific). The blood samples were allowed to stay undisturbed for 2 hours at room temperature and stored at 4°C over night. (Miller *et al*., 1998, Reuveny *et al*., 2001, Gaur *et al*., 2002). The experimental parameters are summarized in the table below.

Table 1: Immunization studies of plant derived PA in mice

Group #	Group	# of animals	Route of Administration	Dose of PA	Volume
1	PA purified from tobacco with adjuvant in (Alhydrogel)	5	Subcutaneous	5µg	200µl
2	PA purified from tobacco without adjuvant	5	Subcutaneous	5µg	200µl
3	Std.PA with adjuvant (Alhydrogel)	5	Subcutaneous	5µg	200µl
4	Std.PA without adjuvant	5	Subcutaneous	5µg	200µl

5	Crude extract of plant expressing PA with adjuvant (Alhydrogel)	5	Subcutaneous	5µg	200µl
6	Crude extract of wild type plant with adjuvant (Alhydrogel)	5	Subcutaneous		200µl
7	PBS only	5	Subcutaneous		200µl

ELISA to detect the anti-PA IgG antibodies in the serum samples

96 well microtiter ELISA plates were coated with 100ul/well of PA standard at a concentration of 2.0µg/ml in Phosphate buffer saline solution, pH 7.4. The plates were stored overnight 4°C. The plates were washed and blocked with PBS containing 0.1% Tween 20 and 3% skim milk powder. The serum samples from the mouse were serially diluted ranging from 1:100 to 1: 640,000. Plates were incubated with 100ul of diluted serum samples for an hour at 37°C followed by washing with PBS-Tween and Water. The plates were then incubated for one hour at 37°C with 100µl of Goat anti-mouse IgG conjugated with Horseradish peroxidase enzyme (1:5000 dilution). This is followed by washing and addition of 100µl of TMB substrate and incubating the plate for 10min at room temperature. The reaction is finally stopped by adding 50µl of 2M sulphuric acid. The plates are read on the plate reader (Dynex technologies) at 450 nm. Titer values are calculated using a cut off value equal to an absorbance difference of 0.5 between

immunized and unimmunized mice. (Quinn *et a*l., 2002, Schneerson *et al*., 2003, Pezard *et al*., 1995, Gaur *et al*., 2002).

RESULTS

Construction of pLD-5'UTR-HisPA83 vector for tobacco chloroplast transformation

The 6Xhistidine tag and the factor Xa site with Nde I and Xho I restriction sites were introduced N-terminal to *pag* using polymerase chain reaction. PCR amplified product of size app. 370 bp with Nde I restriction site at the 5'end and Xho I restriction site at the 3' end was obtained by performing site directed mutagenesis. The PCR product has the Histag, Factor Xa protease cleavage site, partial *pag* sequence as shown in fig 1a. The PCR product was purified using PCR purification kit (Qiagen). Phosphate groups were added to the PCR fragments to the 5' ends of both strands and cloned into EcoRV digested and dephosphorylated p-Bluescript plasmid vector. The transformed colonies were selected based on blue-white selection. Among the white colonies picked, 90% contained the plasmid with the ligated PCR product. The ligated plasmid designated as pBsk-*pag*1 was isolated using miniprep and the PCR product was then sequenced. The sequence was compared with sequence sent by Dr. Bhatnagar which is currently available in NCBI data base with accession number AY700758. The PCR product was isolated digesting the ligated pBsk-*pag*1 with NdeI and XhoI. The PCR product was then cloned into pCR2.1 vector containing the 5'UTR as shown in fig1B and is designated as pCR2.1-*pag*1. (5"UTR is a regulatory sequence derived from the plant psbA gene that helps in transcription enhancement due to the presence of promoter in the sequence and it is a light and developmentally regulated translation enhancer). The pCR 2.1 vector

containing the 5'UTR and the PCR product was digested with Kpn I and Hind III (fragment size app.600bp) and cloned into p-Bluescript-*pag*, designated as pBsk-*pag*2 as shown in Fig1C. Finally the p-Blue script containing the 5'UTR, His tag and *pag* was digested with EcoRV and Xba I (fragment size app.2.5kb) and cloned into tobacco universal vector pLD-ctv, designated as pLD-VK1 as shown in fig1D. The pLD vector contains the homologous recombination sequences (flanking sequences) that allowed the homologous recombination of the gene cassette (*aadA*, 5'UTR,His PA) in between the *trnI* and *trnA* of the chloroplast genome (Daniell *et al*., 2001a). Downstream to the trnI, the vector provided the constitutive 16S rRNA promoter, which regulates the expression of *aadA* gene (aminoglycoside 3' adenyltransferase) that confers resistance to spectinomycin-streptomycin and the His PA gene encoding the protective antigen (Goldschmidt-Clermont, 1991). Downstream to the *trnA*, the vector contains the 3'UTR which is a terminator and transcript stabilizer derived from psbA gene.

Fig 1A

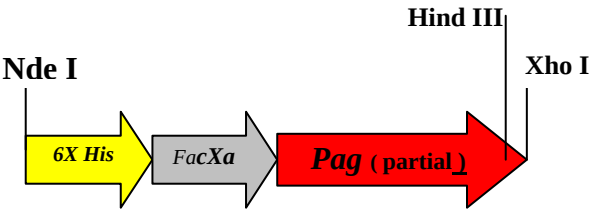


Fig 1B

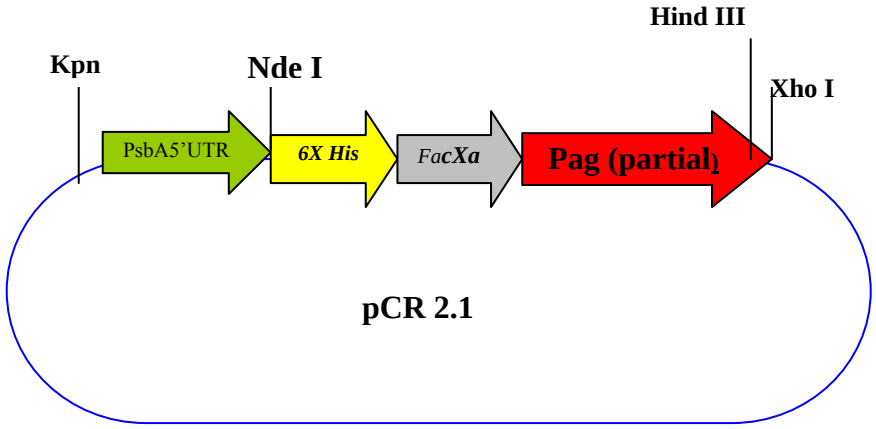


Fig 1C

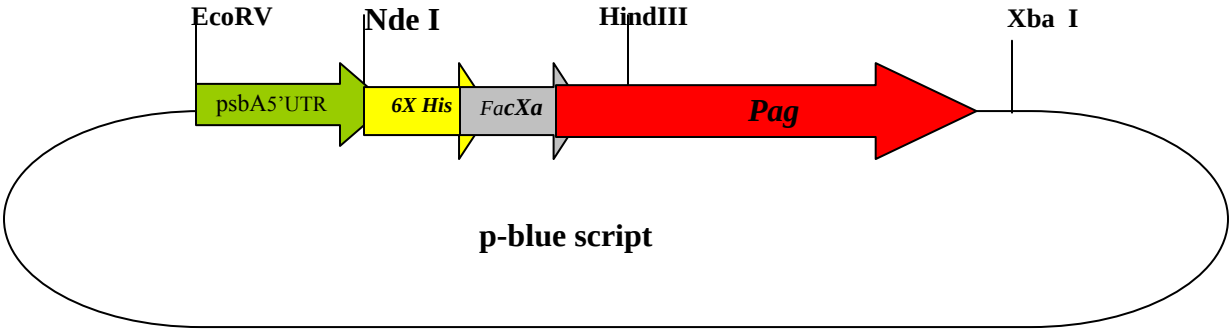

EcoRV
Nde I
HindIII
Xba I
psbA5'UTR
6X His
FacXa
Pag
p-blue script

Fig 1D

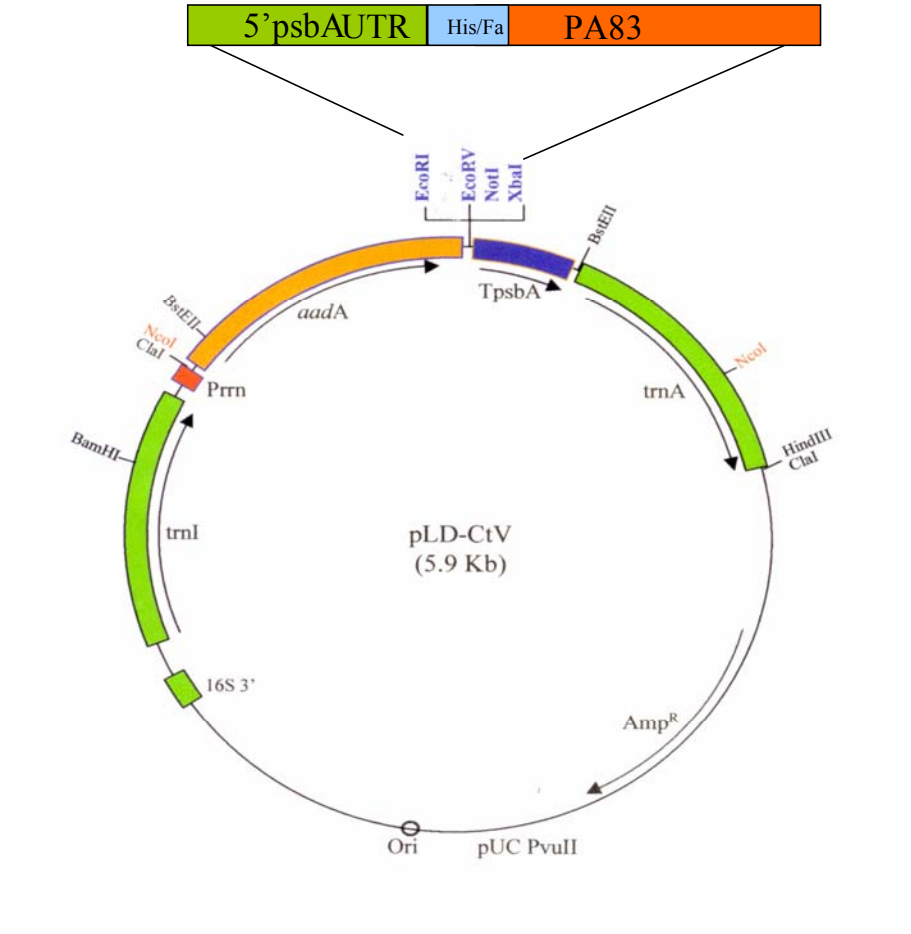

5'psbAUTR
His/Fa
PA83
EcoRI
EcoRV
NotI
XbaI
BstEII
TpsbA
aadA
BstEII
NcoI
ClaI
Prrn
trnA
NcoI
HindIII
ClaI
BamHI
trnI
pLD-CtV
(5.9 Kb)
16S 3'
Amp^R
Ori
pUC PvuII

Figure 1: Schematic steps to clone pLD-5'UTR-HIS-PA83

1A: PCR product showing 6X Histidine tag, factor Xa protease site, *pag* (partial) with Nde I and XhoI restriction sites.
1B: Cloning of PCR product into pCR2.1 using NdeI and XhoI (pCR2.1-*pag*1).
1C: Cloning of pCR2.1 5'UTR into p-Blue script containing *pag* using kpn I and Hind III restriction sites (pBsk-*pag*2).
1D: Cloning of *pag* with 5'UTR into pLD-Ctv using EcoRV and XbaI restriction sites (pLD-VK1).

Construction of pLD-5'UTR-HisPA63 vector for tobacco chloroplast transformation

This vector was designed to knock out the sequence encoding the N-terminal 20kDa portion of PA83 protein. This PCR amplified product of size app. 190 bp has been obtained with Nde I restriction site at the 5'end and Nco I restriction site at the 3' end as shown in fig 2A. The PCR product has the Histag, Factor Xa protease cleavage site and the partial *pag* sequence. The PCR fragment was purified using PCR purification kit (Qiagen). Phosphate groups were added to the PCR fragment, to the 5' ends of both strands and cloned into EcoRV digested and dephosphorylated p-Blue script plasmid vector and is designated as pBsk-*pag*63-1. The transformed colonies were selected based on blue-white selection. Among the white colonies picked, 90% contained the plasmid with the ligated PCR product. The p-Bluescript containing PCR fragment was sequenced. The p-Blue script containing the PCR product was digested with NdeI and NcoI and cloned into plasmid p-Blue script containing the 5'-UTR-His and *Pag* (full size), designated as pBsk-*pag*63-2 fig 2b.The ligated plasmid was digested with EcoRV and Xba I to clone into pLD-CtV, designated as pLD-VK2 as shown in fig 2c.All the plasmids are stored at –20ºC.

Fig 2A

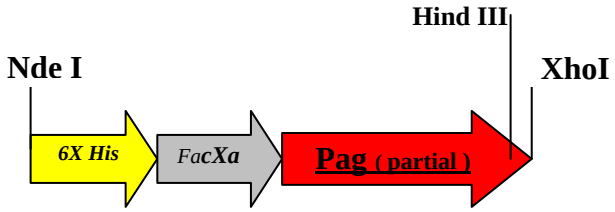


Fig 2B

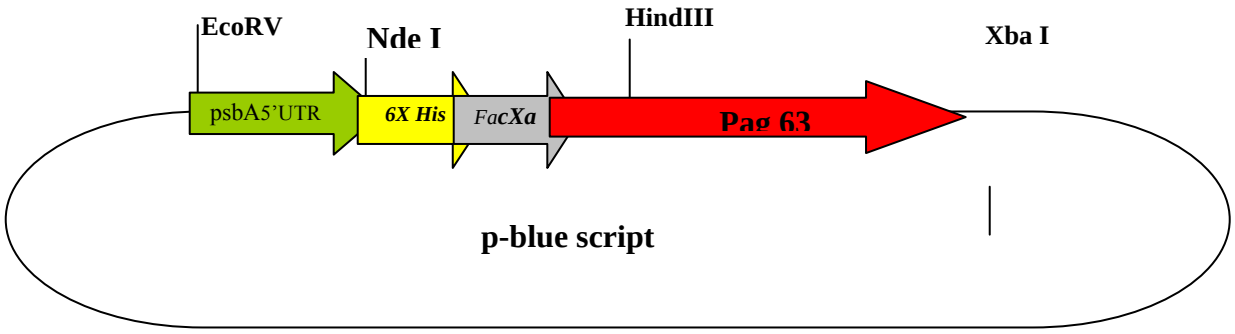


Fig 2C

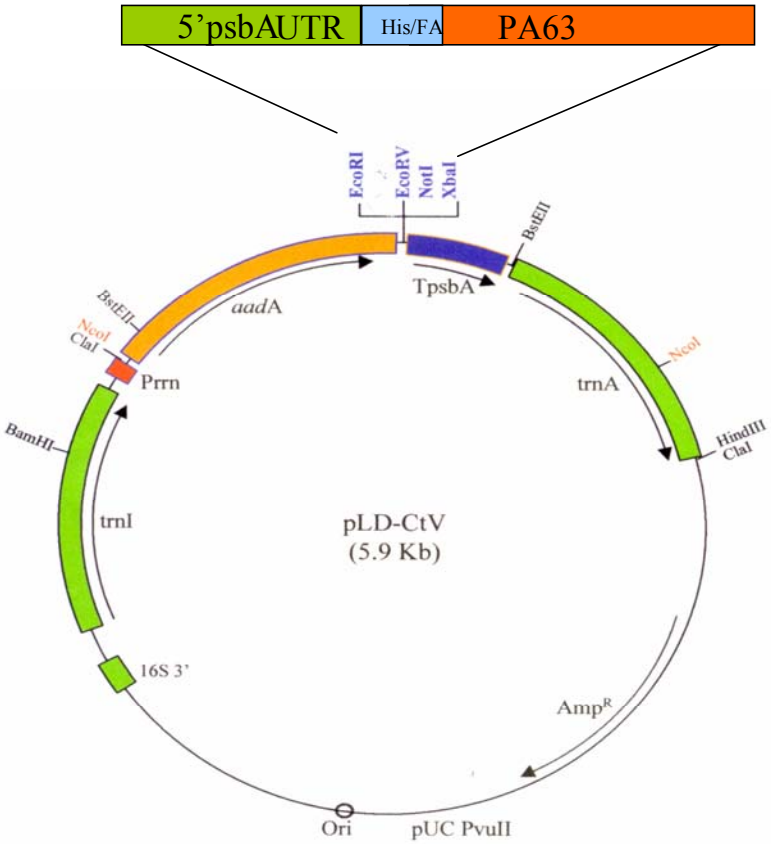


Figure 2: Schematic steps to clone pLD-5'UTR-HIS-PA63

2A: PCR product showing 6X histidine tag, Factor Xa site, NdeI and Nco I restriction sites.

2B: Cloning of PCR product into p-Blue script containing *pag* using NdeI and NcoI restriction sites (pBSk-*pag*63-2).

2C: Cloning of *pag* 63 with 5'UTR into pLD-Ctv using EcoRV and XbaI.

PCR Analysis of Confirmation of Chloroplast Integration of transgenes

After bombardment of tobacco leaves with gold particles coated with plasmid DNA (pLD-5'UTR-HisPA83/ pLD-5'UTR-HisPA63), about 5 shoots appeared after a period of 5-6 weeks. There were 3 possibilities for obtaining shoots on the selection media: chloroplast transgenic, nuclear transgenic, or mutant shoots. Spontaneous mutation of the 16S rRNA gene, which confers resistance to spectinomycin in the ribosome, could allow plants to grow on spectinomycin without integration of the gene cassette. The *aadA* gene in the gene cassette confers resistance to spectinomycin. True chloroplast transformants were distinguished from nuclear transformants and mutants by PCR. Two primers, 3P and 3M, were used to test for chloroplast integration of transgenes (Daniell *et al*., 2001a). 3P primer landed on the native chloroplast DNA in the 16S rRNA gene. 3M landed on the *aadA* gene as shown in fig 3a. Nuclear transformants were eliminated because 3P will not anneal and mutants were eliminated because 3M will not anneal. The 3P and 3M primers upon chloroplast integration of transgene will yield a product of 1.65kb size fragment as shown in figure 3b, 3c.

The Integration of the *aadA*, His+PA83gene and *aadA*, His+PA63gene, were additionally tested by using the 5P and 2M primer pair for the PCR analysis . The 5P and 2M primers annealed to the internal region of the *aadA* gene and the internal region of the *trnA* gene respectively as shown in fig 3A (Daniell *et al*., 2001a). The product size of a positive clone is of 3.9 kb for PA83 and 3.4 kb for PA63, while the mutants and the control do not show any product. Figure 3D shows the result of the 5P/2M PCR analysis After PCR analysis using both primer pairs, the plants were subsequently transferred through different rounds of selection to obtain a mature plant and reach homoplasmy.

3A

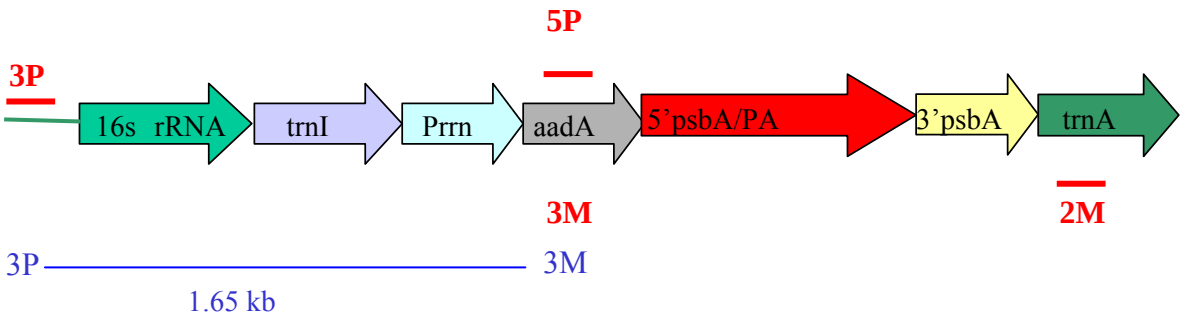


Fig 3B

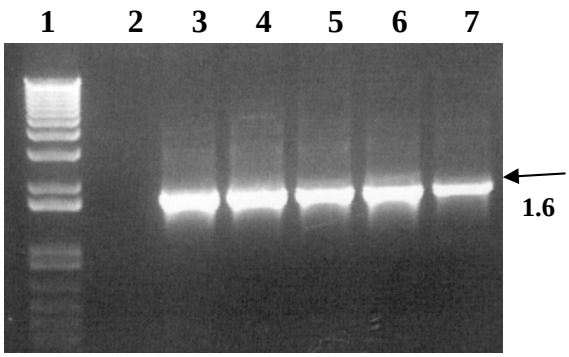


Fig 3C

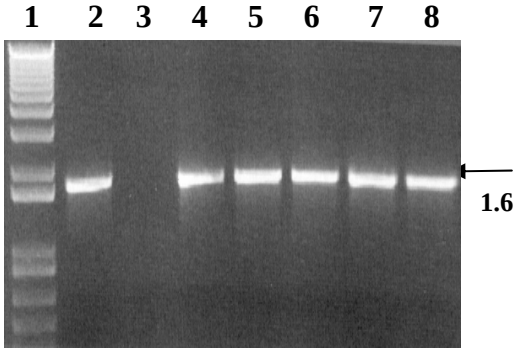


Fig: 3D

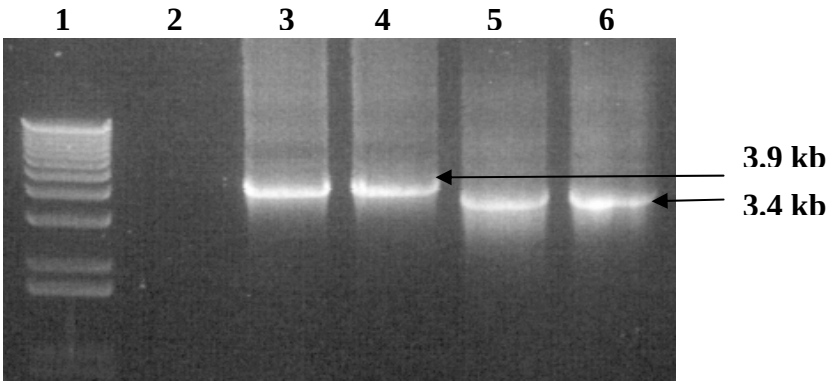


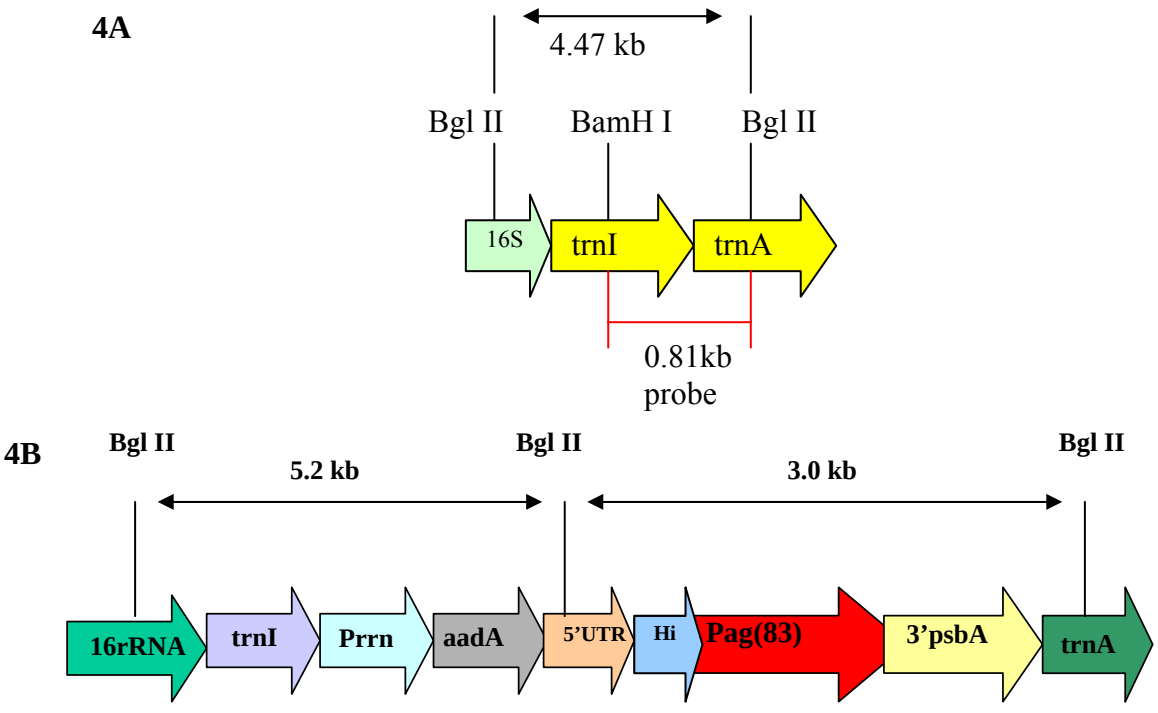
Figure 3: PCR analysis of Wild type and putative transformants of pLD-5'UTR-HisPA83/PA63

3A) PCR using specific primers land within the native chloroplast genome (3P/3M) to yield a 1.65 kb product. **3B**: **Lane1**: 1 kb DNA Ladder, **Lane2**: Wild type, **Lane 3-6**: Transgenic lines pLD-5'UTR-HisPA83, **Lane 7**: Positive control (Interferon clone). **3C**: **Lane1**: 1 kb DNA Ladder, **Lane2**: Positive control (Interferon clone), **Lane 3:** Wildtype**, Lane 4-8**: Transgenic lines pLD-5'UTR-HisPA63. **3D**. **Lane1:** 1Kb plus DNA ladder, Lane 2: Wild type, Lane 3,4: Transgenic lines pLD-5'UTR-HisPA83, Lane 5,6: Transgenic lines pLD-5'UTR-HisPA63.

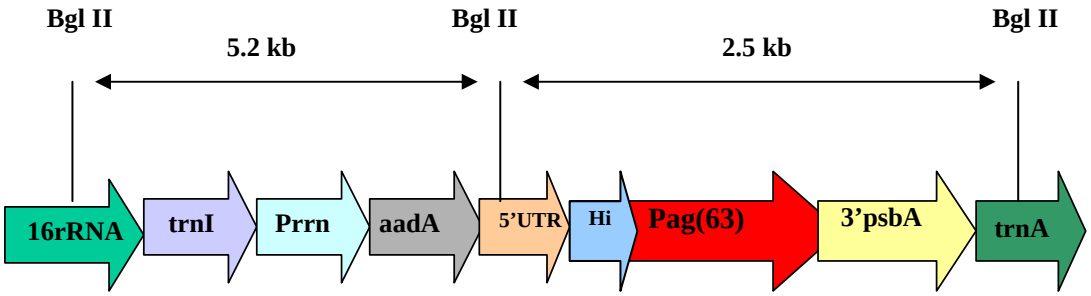
Southern Analysis of transgenic plants

The plants that tested positive for the PCR analysis were moved through three rounds of selection and were then tested by Southern analysis. The DNA of the full regenerated clones growing in jars (third selection) was extracted and used for the Southern analysis. The flanking sequence probe of 0.81kb in size allowed detection of the site-specific integration of the gene cassette into the chloroplast genome; this was obtained by double digesting the pUC-ct vector that contained the *trnI* and *trnA* flanking sequences (fig.4A) with BamHI and Bgl II (Daniell *et al*., 2001a). Figures 4b and 4c show the BglII sites used for the restriction digestion of the plant DNA for pLD-His-

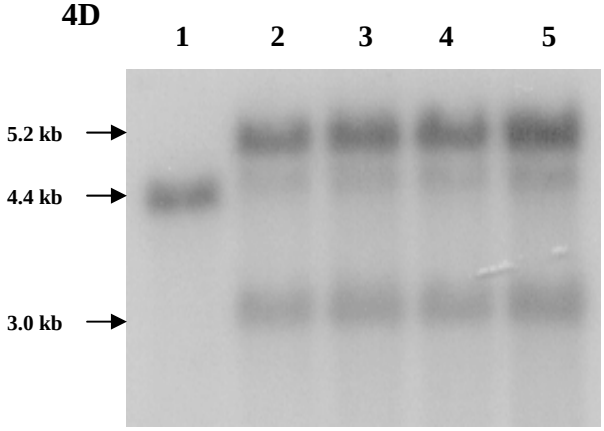
PA83 and pLD-His-PA63 constructs respectively. The transformed chloroplast genome digested with BglII produced fragments of 5.2 kb and 3.0 kb for pLD-PA83 and 5.2 kb and 2.5 kb for pLD-PA63 constructs (Fig 4D), while the untransformed chloroplast genome that had been digested with BglII formed a 4.47kb fragment. The flanking sequence probe can also show if homoplasmy of the chloroplast genome has been achieved through the three rounds of selection. The plant expressing PA83 showed slight degree of heteroplasmy as few of the wild type genomes were not transformed. Whereas the plant expressing PA63 showed homoplasmy. The gene specific probe of size approx.0.52 kb was used to show the specific gene integration producing a fragment of 3kb and 2.5 kb for PA83 and PA63 respectively as shown in fig 4F.



4C

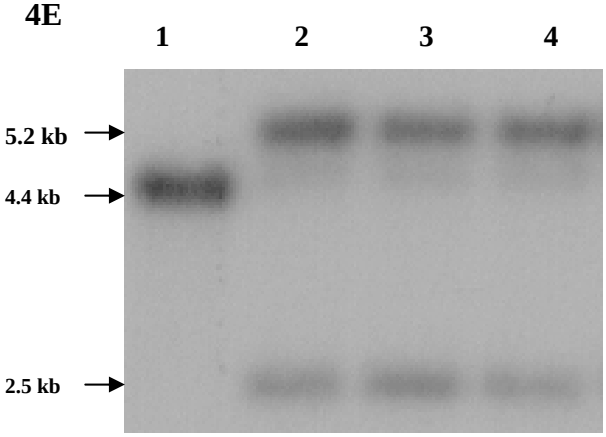


4D



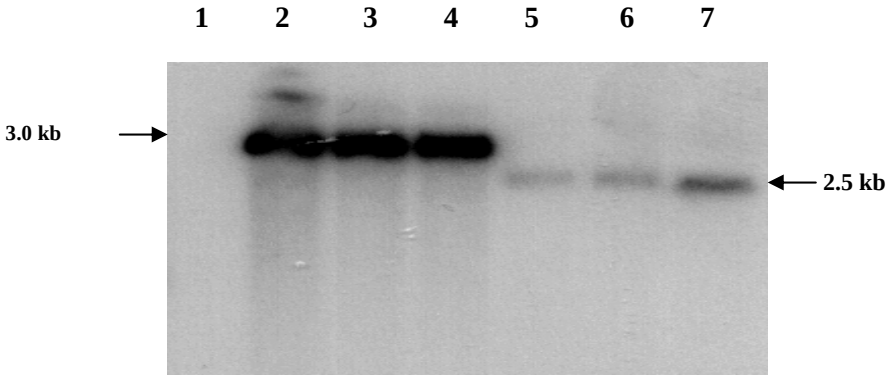
Flanking sequence probe

4E



Flanking sequence probe

4F



Gene Specific probe

Figure 4: Southern blot analysis of pLD-5'UTR-His-PA83 and pLD-5'UTR-His-PA63

Schematic diagram of expected products from digestion of (A) wild type untransformed plant (B) Plants transformed with pLD-5'UTR-His-PA83 (C) pLD-5'UTR-HisPA63 showing homoplasmy. D) Southerns with flanking sequence probe in pLD-5'UTR-His-PA83 transgenic lines showing slight heteroplasmy: Lane1: Wild type, Lane 2: Transgenic line 1, Lane 3: Transgenic line 2, Lane 3: Transgenic line 3.E) Southerns with flanking sequence probe showing homoplasmy in pLD-5'UTR-His-PA63 transgenic lines: Lane1: Wild type, Lane 2: Transgenic line # 1, Lane 3: Transgenic line # 2, Lane 3: Transgenic line # 3. F) PA gene specific probe showing the presence of pag in the transgenic plants: Lane 1: Wild type, Lane 2,3,4: pLD-5'UTR-His-PA83 transgenic lines, Lanes 5,6,7: pLD-5'UTR-His-PA63 transgenic lines

Immunoblot analysis

Crude protein extract of 2ug, was loaded in each well of the SDS-PAGE. The western blot analysis with anti-PA monoclonal antibody revealed the full-length 83 kDa protein. The 83 kDa PA has several protease cleavage sequences like furin cleavage sites trypsin and chymotrypsin cleavage site within the amino acid sequences. The cleavage of these protease sites would result in a 63 kDa, a 47 kDa, and a 37kDa band for furin, trypsin and chymotrypsin site respectively. The absence of these bands reveals that the full-length protein is intact within the chloroplast (See Fig 5A). Occasionally, break down of protein was observed due to repeated freezing and thawing. The supernatant samples from wild type plants did not show any band indicating that anti-PA antibodies did not cross-react with any plant protein in the crude extract. Immunoblot analysis of crude extracts of plants expressing PA63 revealed lower levels of expression of PA63. Each lane was with loaded with 30ug of protein crude extract .The blot shows the relative expression levels of PA83 and PA 63 (Fig5B).

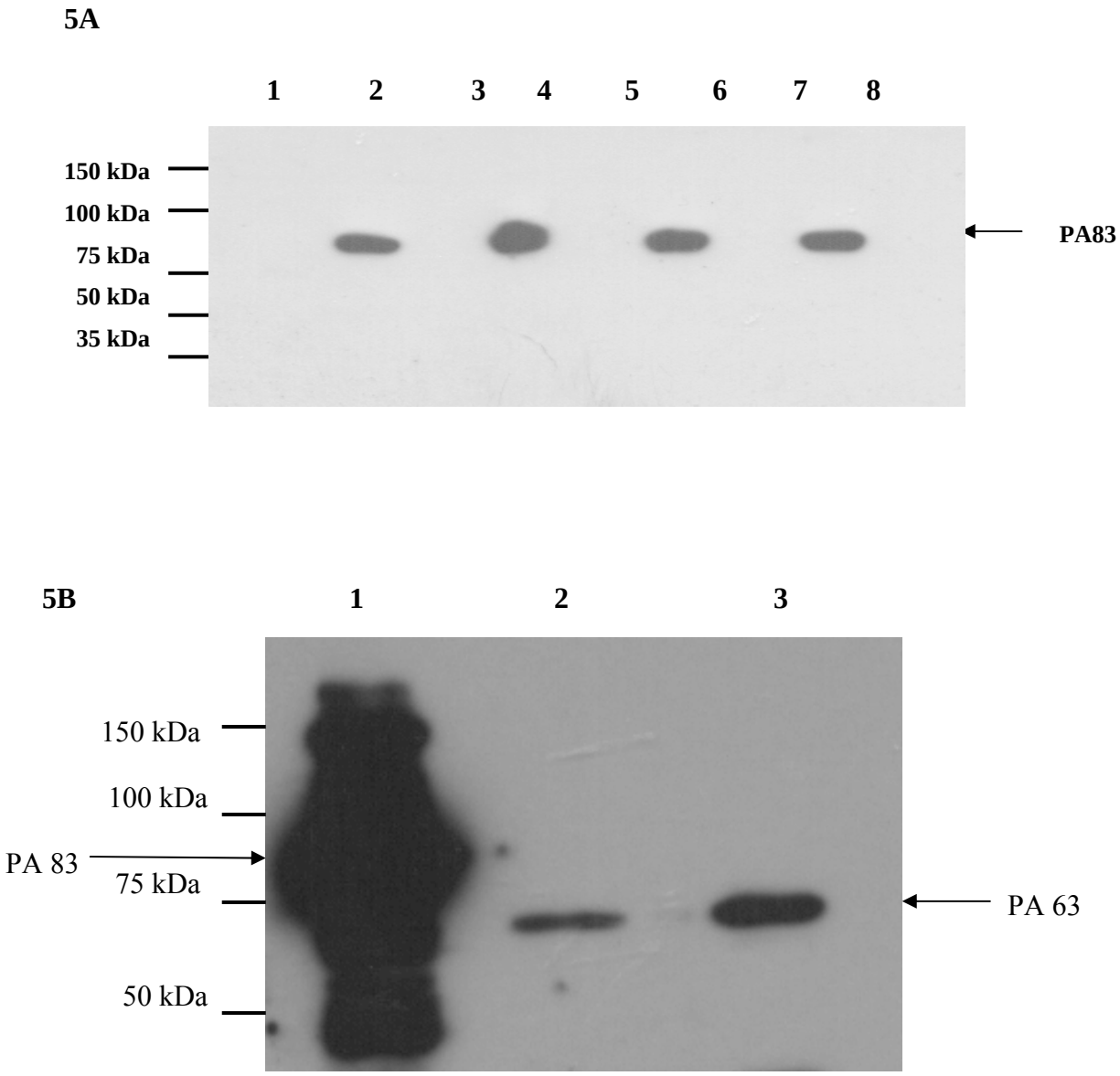


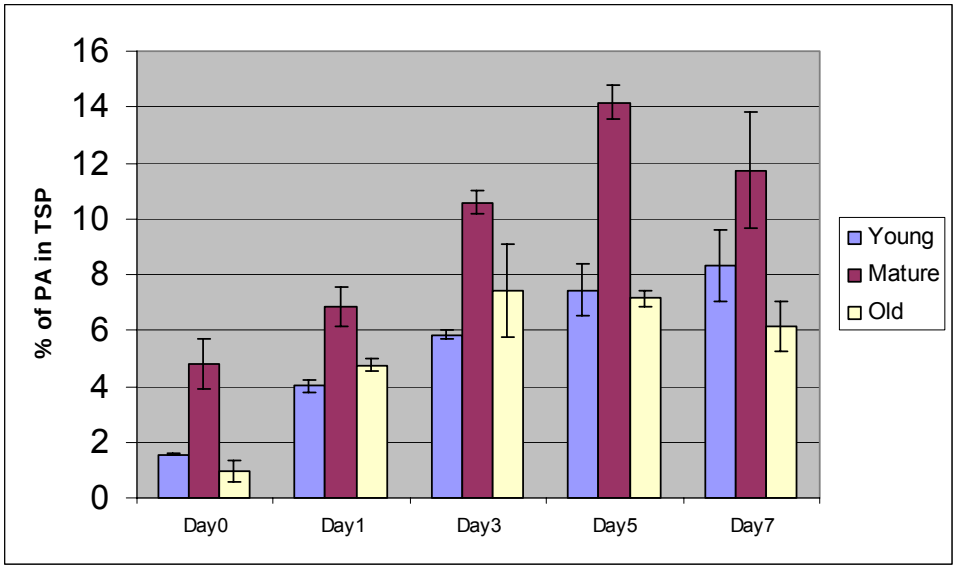
Figure 5: Immunoblotting analysis of crude extracts of plants expressing PA83/Pa63 5A: Western blotting demonstrating the expression of PA in transgenic plant crude extracts: **Lane1**: Wildtype, **Lane 2**: 100ng Standard, **Lane 4**: Transgenic line5, **Lane** 6: Transgenic line 7, **Lane 8**: Transgenic line 8, **Lane 3,5,7**: Empty.**5B: Western blotting demonstrating the expression of PA63 in transgenic plant crude extracts in comparision with expression of PA83**: Lane1: Crude extract of transgenic line expressing PA83, Lane 2: Transgenic line #1expressing PA63+His, Lane3: Transgenic line #2 expressing PA 63+His.

Protein quantification using ELISA

The standard curve has been obtained using different dilutions of purified PA83 produced in *Bacillus anthracis*. The dilutions were made in coating buffer. To estimate the expression levels of recombinant PA83 protein, the known concentrations of transgenic plant's total soluble protein was compared with that of the known concentrations of purified PA antibody complex (standard curve). The PA83 protein expression levels of pLD-5'UTR-PA83 plant of T_0 generation reached up to 4.48% of total soluble protein (TSP) in mature leaves under the regular illumination conditions (16hrs light and 8hrs dark)(Fig 6A) while the pLD-5'UTR-PA63 plant of T_0 generation reached up to 0.8% TSP (Fig.6B). The *psb*A regulatory sequences, including the promoters and UTRs, have been shown to enhance translation and accumulation of foreign proteins under continuous light (Fernandez *et al*., 2003). Therefore, the pLD-5'UTR-PA83 transgenic lines were exposed to continuous light (24hrs) and expression patterns were observed on day1, 3,5 and 7(Fig 6A). The PA83 expression levels reached up to maximum of 14.17 % of total soluble protein (TSP) in mature leaves at the end of day 5 and the expression levels declined to 11.73 % TSP on day 7 (Fig 6A). The maximum expression levels in mature leaves upon 5 day continuous illumination reached up to 1.84 mg per gram of fresh leaf weight. The maximum PA83 expression was observed in mature leaves compared to young and old leaves (Fig 6A). The large contribution of PA from the mature leaves was due to the high number of chloroplasts in mature leaves and the high copy number of chloroplast genomes (up to 10,000 copies per

cell) resulting in very high levels of PA expression in mature leaves. The decrease in PA expression in old plants could be due to degradation of the proteins due to senescence.

6A



6B

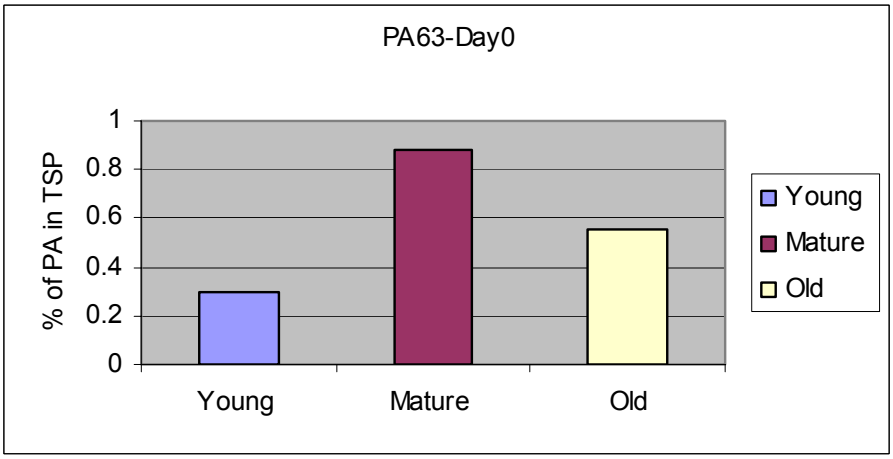


Figure 6: Quantification of expression of levels of PA83 and PA63 in T_0 generation

A) Expression levels in %TSP of PA83 expressing leaves (Young, Mature and Old) under normal and continuous illumination observed for 0-7 days. B) Expression levels in %TSP of PA63 expressing plants under regular illumination conditions (16hs Light and 8hrs dark).

Purification of chloroplast derived PA

The leaves were collected from the transgenic plant expressing PA83.The leaf material (6grams) was ground into fine powder in motor pestle using liquid nitrogen. The ground material was taken into falcon tube and the material was churned in plant extraction buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g NaN_3, 0.1% Tween 20, and 5mM PMSF adjusted to pH 9.6). The supernatant was collected after centrifugation at 10,000 rpm for 15min. The supernatant was loaded onto the sample loop of Akta prime machine. The sample (supernatant of leaf extract) volume was made sure not to exceed more than sample loop volume. First the column was primed with Elution buffer to get rid of salts or any proteins in the resin followed by washing with water. Then the column was applied with 100mM Nickel Sulfate solution followed by washing with water to get rid of the excess of Nickel Sulfate. After Nickel application, binding buffer was passed through the column to aid in binding of proteins. This was immediately followed by sample application. The excess non-specific proteins that are binding to the column was washed with water. During this wash, the flow through was collected to check if the protein if interest was being washed away. Finally the PA83 protein with Histag was eluted using elution buffer containing 0.5M Imidazole in a gradient manner (Fig 7A). The protein eluted at 30% Imidazole gradient. The pooled fractions containing purified plant derived PA83 was dialyzed against ice-cold Phosphate buffer saline (PBS) (200 volumes) over night to displace the imidazole in the elution fractions with the PBS. Finally the protein was loaded onto the 50K MW protein concentrator column to obtain concentrated

protein. The concentrators were centrifuged at 3000 rpm until the volume reached 1000ul.The Column flow through was saved to check for presence of proteins. To visualize the purified protein, SDS-PAGE was run with purified fractions. Fig 7Bshows the comassie staining of the purified protein, flow through and with the crude leaf extracts of PA83 expressing and wild type plant. Upon staining with comassie, the gel shows the purified PA83 protein and also shows that absence of PA83 protein being washed away in the flow through.

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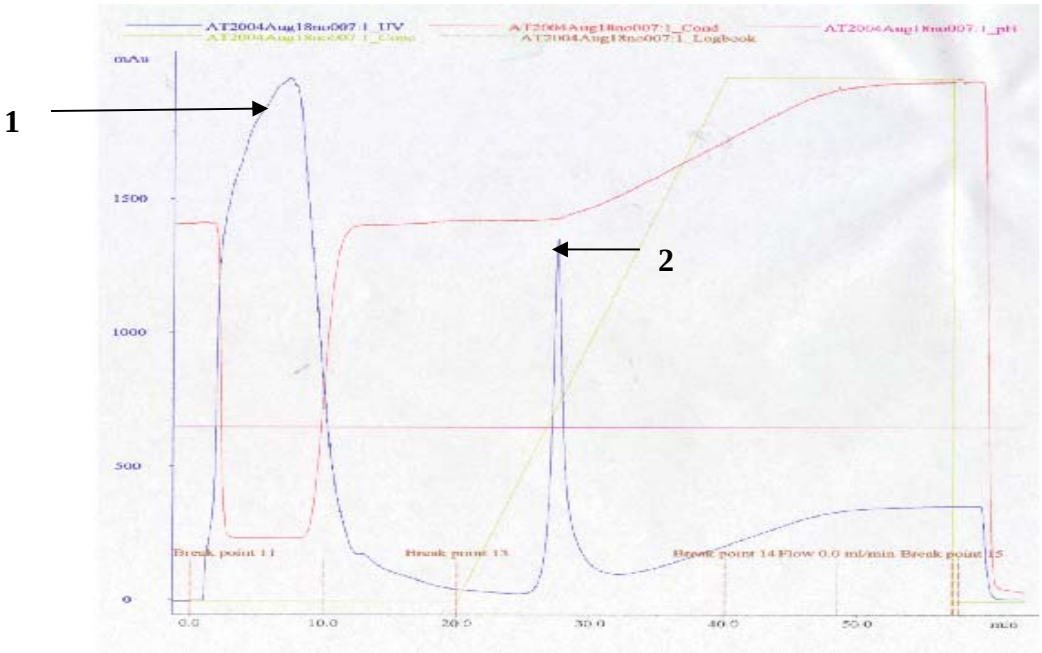
Fig 7A



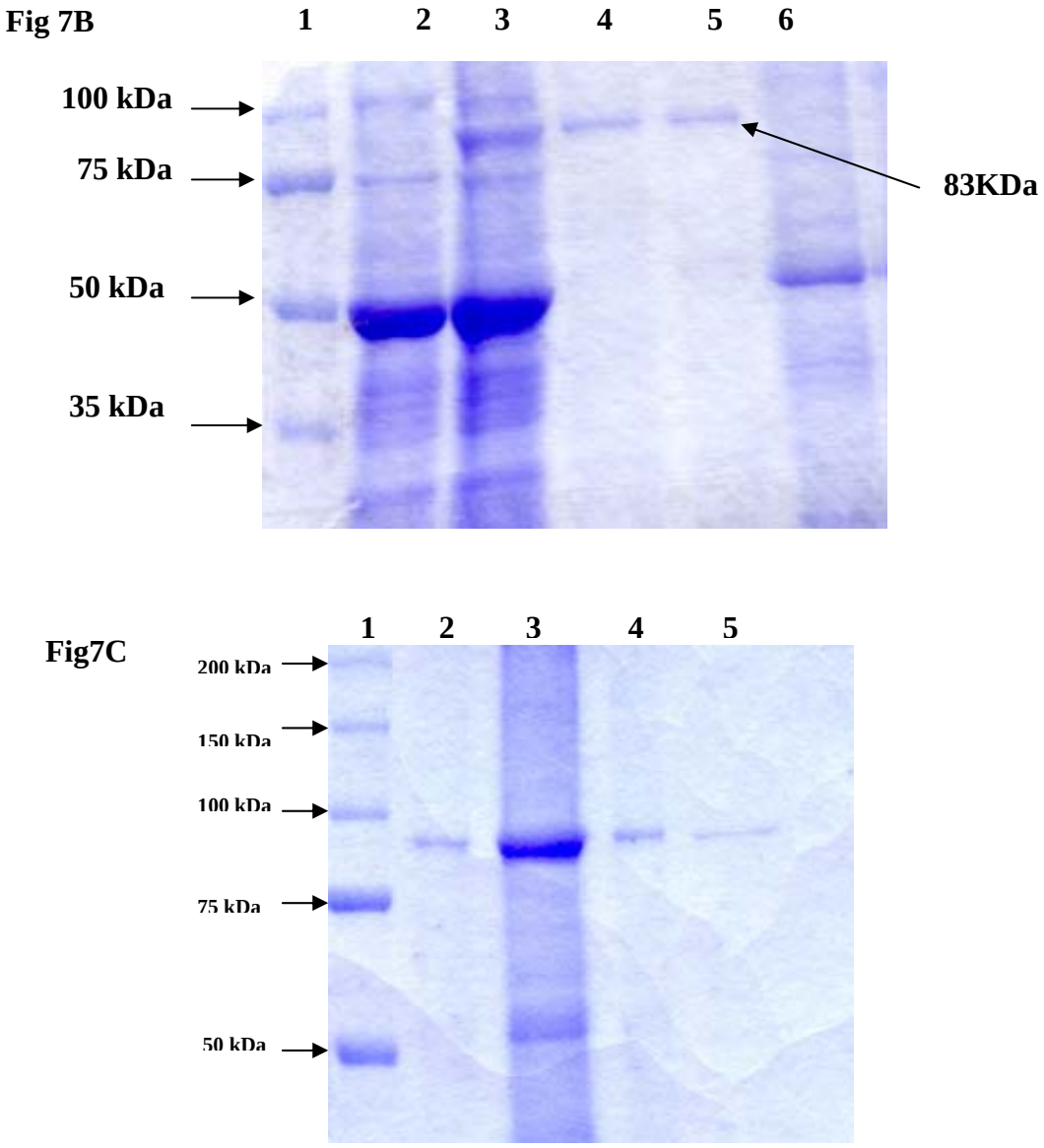


Figure 7: Protein purification
7A: 1.Points to the wash Peak, 2. Points to the Elution Peak, Elution peak obtained using gradient of 0.5M Imidazole**. Fig7B: Comassie staining of the proteins in crude extract and purified protein: Lane1**: Protein plus precision Ladder, **Lane2**: Wild type leaf crude extract, **Lane3**: Crude extract of transgenic plant expressing PA83, **Lane 4,5**: Purified chloroplast derived PA, **Lane6**: Flow through collected during purification.
7C: Lane1: Ladder, **Lane3**: Concentrated protein, **Lane5**: Purified protein (before concentrating), Lane2 &4: Over flow from lane3.

Macrophage lysis assay

Supernatant samples from crude extracts of plant leaves expressing PA83 with Histag and PA63 with His Tag, purified chloroplast derived PA83 with His tag were tested for functionality *in vitro*. The functionality of the chloroplast-derived PA83 was determined by its ability when PA83 gets cleaved by furin protease into PA63 followed by internalization of the lethal factor and cause lysis of cultured mouse macrophages. The percentage macrophage viability was determined using the ability of live but not dead cells to reduce a water-soluble yellow dye, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to an insoluble purple formazan product. The transgenic plants were shown to produce fully functional PA83 (Fig.8). A comparative assay was done on the supernatant samples of crude extract of PA83 with his tag, crude extract of PA63 with his tag, purified chloroplast derived PA with his tag (20ug/ml) and the PA purified from *Bacillus anthracis* of known concentration (20ug/ml) as positive control. Crude extract of wild type tobacco plant and plant extraction buffer were used as negative controls. The PA83 binds to the anthrax toxin receptor on the macrophages and gets cleaved to PA63 with the furin like proteases and allows the lethal factor to bind to the N-terminal of PA63 followed by internalization. The functional assays revealed that the crude extract of PA83 with his tag and the purified chloroplast derived PA83 with His tag were able to lyse the mouse macrophages on combining with lethal factor. It was also determined that the crude extract of plant leaves expression PA83 and purified chloroplast derived PA83 contained about 20ug/ml of functional PA. The PA 63 with his tag acted as a negative control because of the absence of furin protease cleavage site that

was necessary for the lethal factor to bind to PA63 upon cleavage. The plant protein extraction buffer and the crude extract of wild type leaf did not showed no activity proving the absence of toxicity due to the buffer and plant extract components.

Fig 8

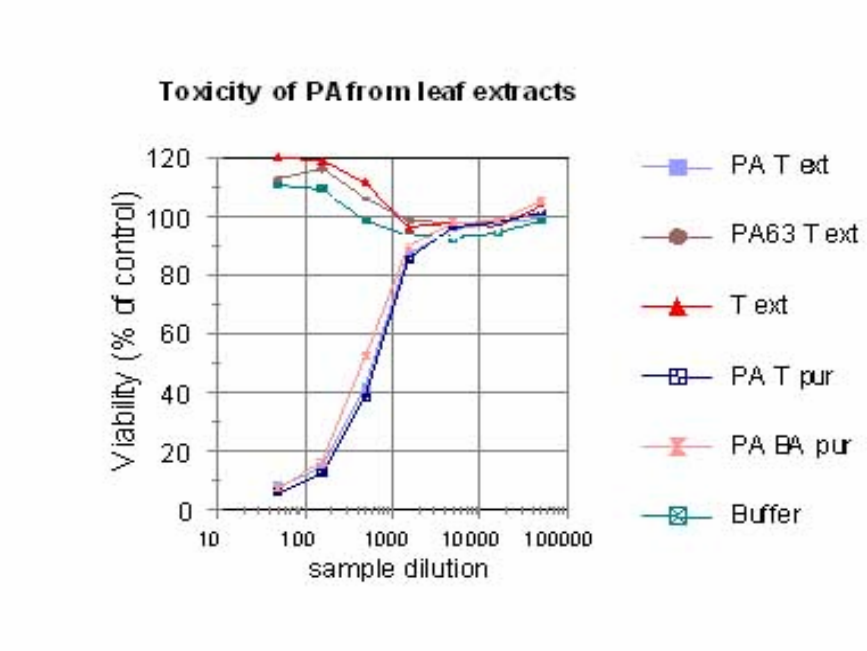


Figure 8: Macrophage lysis assay

The cytotoxicity of PA-LF on macrophage cell lines was analyzed on various sources of PA. Supernatant samples of plant leaves extracts expressing PA83 with Histag (PA T ext), and PA63 with Histag (PA63 Text), Wild type plant crude extract (T ext), Purified chloroplast derived PA (PA T pur), Purified PA derived from *Bacillus anthracis* (PA BA pur) and plant protein extraction Buffer.

Immunization in mice

7 groups of mice each containing 5 mice were injected subcutaneously with antigen doses as per the table given in materials and methods section. The doses were given on day 0,14,28. Blood was collected from retro orbital plexus on 14th day after the

final dose. Serum was extracted and performed ELISA for the antibody titers. The results of the immunological studies are summarized in the Fig 9. All the groups injected with either purified PA83 or crude extract of plant expressing PA83 responded with significant IgG anti-PA levels. The Group of mice that received purified chloroplast derived PA83 with adjuvant showed a maximum IgG titers of 1:320,000 and the group that received PA83 from *Bacillus antharcis* with adjuvant also reached maximum IgG titers of 1:320,000. This observation that chloroplast derived PA83 and PA derived from *Bacillus anthracis* elicit comparable immune responses shows that the plant derived PA83 has been properly folded and functionally similar to that of PA derived from *Bacillus antharcis*. The group that received purified plant derived PA without adjuvant showed titers ranging from 1:40,000 to 1:80,000. The group that received PA derived from *Bacillus anthracis* without adjuvant showed titers 1:80,000 to 1:160,000. Difference in the titer values of the groups that received PA83 with adjuvant and the group without adjuvant was due to the slow release of PA83 bound to the adjuvant so that the effect of PA on the immune system lasts for longer time. The group that received transgenic plant crude extracts expressing PA83 with adjuvant showed IgG titers ranging from 1:40,000 to 1:80,000. The group that received wild type plant leaf crude extract showed no anti PA IgG titer value, indicates the specificity of the immune response.

Fig: 9

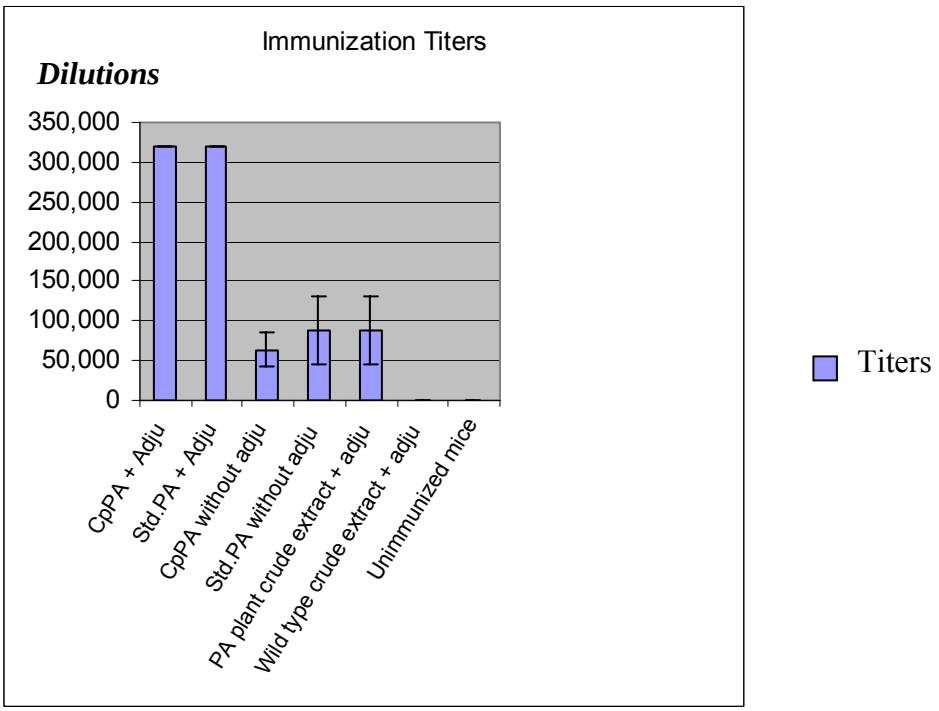


Figure 9: ELISA for detection of IgG Antibody Titers in Serum samples collected from mice on Day 14 after final booster

Comparison of immune responses in mice injected with Chloroplast derived PA (CpPA), Std.PA derived from *Bacillus anthracis* with and without adjuvant, Subcutaneous/Oral.

Immunoblotting with serum of mice immunized with purified chloroplast derived PA

The crude extracts of plants expressing PA83 and the *Bacillus anthracis* protective antigen (PA83) were run on SDS –PAGE, transferred onto nitrocellulose membrane. The membrane was incubated with serum at 1:10000 dilution obatined from the mice immunized with chloroplast derived PA83. The serum anti-PA antibodies were

able to recognize the chloroplast derived PA as well as PA derived from *Bacillus anthracis.* The serum antibodies did not cross react with any other plant proteins proving the specificity of the antibodies.

Fig: 10

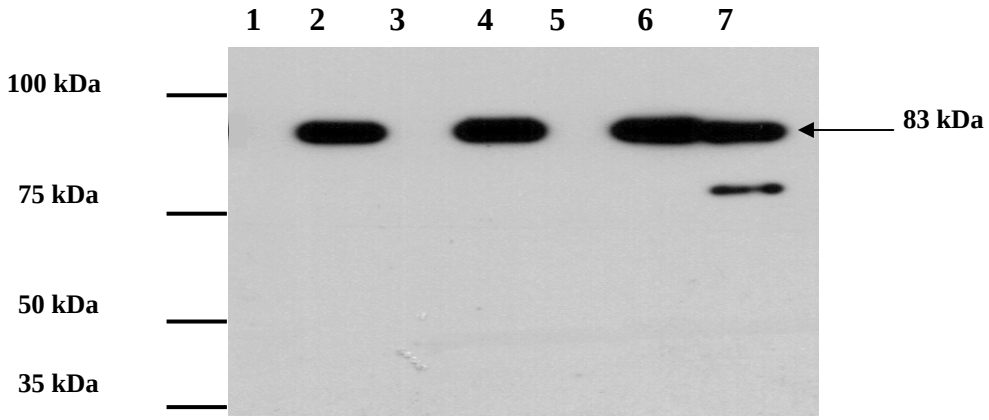


Figure 10: Immunoblotting with serum of mice immunized with purified chloroplast derived PA83

Lane1: Ladder, Lane 2,4,6: Crude extracts of plants expressing PA83, Lane7: PA derived from Bacillus anthracis, Lane3,5: Empty.

DISCUSSION

The pLD-5'UTR-His-PA83/63 vector was derived from the universal transformation vector, pLD-CtV (Daniell H., 2001a). The pLD-5'UTR-His-PA83/63 chloroplast transformation vector containing the aadA gene and the PA83/63 coding

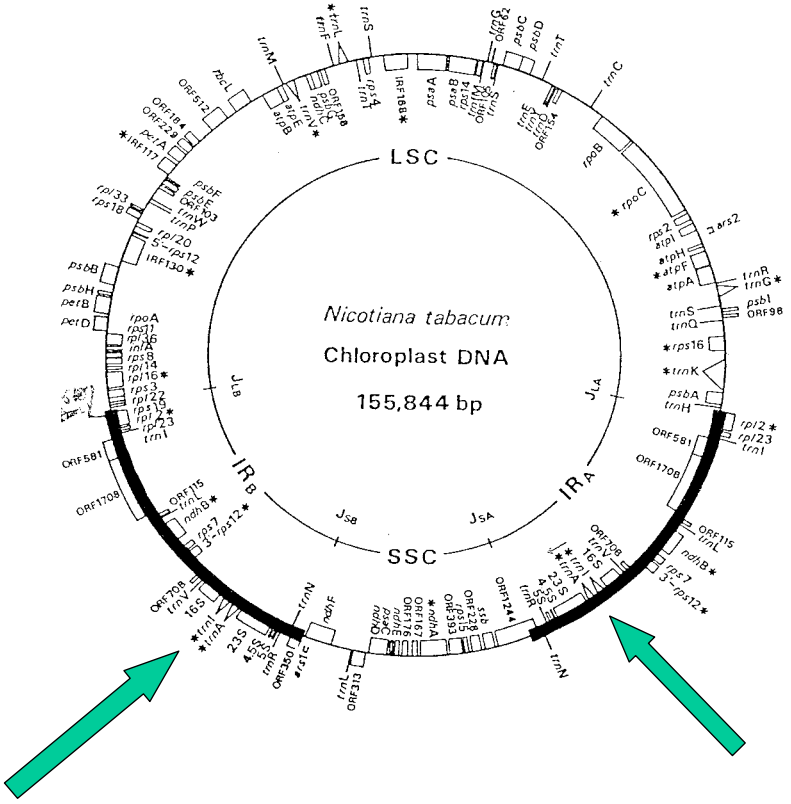


Figure 11: The Chloroplast Genome

Arrows point to trnI and trnA where homologous recombination occurs between the chloroplast and the pLD-5'UTR-His-PA83/63 vector.

region, integrates the transgene cassette into the trnI – trnA region of the chloroplast genome via homologous recombination (Fig: 11). Integration takes place in one of the inverted repeats and is copied into the other inverted repeat via the mechanism of copy correction (Devine and Daniell, 2004). Expression of the PA83/63 recombinant protein in the chloroplast depends on several factors. First, the pLD-5'UTR-His-PA83/PA63 vector is designed to integrate into the inverted repeat region of the chloroplast genome via homologous recombination. The copy number of the transgene is thus doubled when integrated at this site. Increased copy number results in increased transcript levels resulting in higher protein accumulation (Daniell *et al*., 2001a). Second, the 5' and 3' untranslated regions (UTRs) used for the regulation of transgene expression help in enhancing translation of the foreign protein (Eibl *et al*., 1999). Third, homoplasmy of the transgene is a condition where all of the chloroplast genomes contain the transgene cassette. There are 100 to 1000 chloroplasts per cell and 100 to 1000 chloroplast genomes per chloroplast (Daniell *et al*., 2002, Decosa *et al*., 2001, Devine and Daniell, 2004); for optimal production of the recombinant protein and transgene stability, it is essential that homoplasmy be achieved through several rounds of selection on media containing spectinomycin. If homoplasmy is not achieved, heteroplasmy could result in changes in the relative ratios of the two genomes upon cell division. The presence of heteroplasmic condition in a transgenic plant might retrograde back to the wildtype eliminating the transgene in the absence of selection pressure in subsequent generations. The chimeric, aminoglycoside 3' adenyltransferase (aadA) gene, conferring resistance to spectinomycin was used as a selectable marker and its expression is driven by the 16S (Prrn) promoter (Daniell *et al*., 2001a, Svab and Maliga, 1993). Spectinomycin binds the

70S ribosome and inhibits translocation of peptidal tRNAs from the A site to the P site during protein synthesis. The aadA gene codes for the enzyme aminoglycoside 3' adenyltransferase, which transfers the adenyl moiety of ATP to spectinomycin, inactivating it. The pLD-5'UTR-His-PA83/PA63 vector has a functional chloroplast origin of replication, which increases the number of templates for integration into the chloroplast genome (Daniell *et al*., 2004d). Fourth, expression can depend on source of the gene and its' relative AT/GC content. The prokaryotic-like chloroplast favors AT-rich sequences, which reflects the respective tRNA abundance. Therefore the PA bacterial gene having 67% AT are expected to express well in the chloroplast. High expression of synthetic Human Somatotropin (HST), human serum albumin, human interferon-α2b, Human interferon-γ, Insulin like growth factor shows that eukaryotic genes can also be expressed in the plastid (Staub *et al*., 2000, Fernandez *et al*., 2003, Daniell *et al*., 2004c, Leelavathi and Reddy, 2003, Daniell, 2004) however; some eukaryotic genes need to be optimized for chloroplast expression. Genetic engineering of the chloroplast genome to express the PA83/63 with His tag served two purposes, that is to achieve high expression of the foreign protein and for gene containment. The use of the psbA-5'UTR has been shown to be vital in achieving hyper expression of several proteins in the chloroplast. The *psbA* gene encodes for the 32 kDa D1 protein, which is an integral part of Photosystem II. It has been shown that the initiation of psbA mRNA translation in tobacco is light regulated by sequences outside of the coding region (Staub and Maliga, *et al*., 1994, Fernandez *et al*.,2003, Dhingra *et al*.,2004, Watson *et al*., 2004). The 3'UTR region of the psbA gene present in the transgene cassette confers transcript stability. Using mutants generated by sequence deletion and base alteration, it has been

recently demonstrated that the correct primary sequence and secondary structure of the psbA 5'UTR are required for mRNA stabilization and translation (Zou *et al*., 2003).The expression levels in plant leaves expressing PA83 with Histag on day 0 (4.78% in mature leaves) is relatively high compared to PA63 with Histag on day 0 (0.8% in mature leaves). There is a difference in the expression levels between the two constructs. Besides, though the plants expressing PA63 showed flowering, they did not produce seeds. So the plants expressing PA83 only were passed on for the continuous light studies. While PA63 expressing plants were allowed to grow under regular light pattern for them to produce the seeds. Seeds were never produced even after several months. The light-regulated 5'UTR from *psb*A gene, including its promoter, was used to enhance transcription and translation of PA. The light-regulated psbA-5'UTR therefore accounts for both the high expression of PA and the change in expression over 5 days of continuous illumination. The highest percentage of PA83 with his tag in Total Soluble Protein (%TSP) peaked in the mature leaves in day five (14.17%). Percent TSP values depend on the accumulation of all the proteins in the plants cell. By day seven of continuous illumination, plants appeared stressed, having decreased green pigmentation. Interruption of light dark periods therefore probably caused up-regulation of some stress-induced proteins and down-regulation of photosynthetic proteins as well as the PA transgene driven by the psbA-5'UTR. Protein isolation and purification from plants is difficult task, owing to the complexity of the plant system compared to bacterial or yeast systems (Desai *et al*., 2002). The better yield of the desired protein can be obtained by reducing the purification steps. Current purification techniques for plant proteins involve a series of low-efficiency purification steps, which include several ion-exchange and gel-

filtration chromatography columns. With each step the yield of target protein is reduced and the manufacturing costs are increased. It is well established that affinity chromatography is a powerful purification method that can reduce the number of steps and increase the purity of the isolated protein. It takes advantage of the highly specific binding affinity of the target protein toward an immobilized ligand (Hentz *et al*., 1996).The His tag present at the N-terminal end of PA 83 aided in purifying the PA83. Affinity chromatography with resin column carrying Nickel ions binds to the 6X-Histidine chain present N-terminal to PA83. The non specific plant proteins are washed away followed by the elution with gradient of 0.5 M Imidazole. The purified protein was detected in the fractions using ELISA. The functionality of the PA83 with Histag was analysed *invitro* using macrophage lysis assay. The chloroplast derived purified PA is functionally similar to PA83 derived from *Bacillus anthracis*. The PA83 was cleaved by the furin proteases and the PA63 fragment was able to bind to LF, be internalized and lyse the macrophage cells. The mouse macrophage lysis assay showed that there is atleast 20ug/ml of functional PA. The Immunization experiments in mice showed that the PA83 derived from chloroplast was functionally similar to PA derived from *Bacillus anthracis in vivo.* The anti-PA IgG antibody titers obtained in the group of mice injected with chloroplast derived PA83 is similar to that of produced in the mice group injected with PA83 derived from *Bacillus anthracis*. The antibody titers for the mice group injected with purified PA83 with adjuvant was four times higher than the group injected without adjuvant. The studies had clearly proved that the Chloroplast derived PA83 is functionally as good as PA83 derived from *Bacillus anthracis*.

Future studies

Assessment of Immunoprotective properties of transgenic Plant-derived PA83

The mice will have to be tested for the immunoprotective properties. For this the mice will be challenged with lethal toxin of *Bacillus anthracis*. The survival percentage will be evaluated and immunoprotective properties will be determined.

Development of oral delivery immunization protocol

Optimized protocol for the oral delivery of PA will be designed to develop significant IgA mucosal immune response**.**

Development of transgenic carrots expressing PA83

Development of transgenic carrot expressing PA83 will open the door for the oral delivery of the vaccine and develop mucosal immune response. An ideal vaccine for *Bacillus anthracis* should induce both mucosal and systemic protection. If both subcutaneous and oral prove to be immunoprotective, priming both the mucosal and systemic systems may prove not only to be the cheapest way but also the most immunoprotective way of vaccination against any pathogen that attacks both the mucosal and systemic systems.

CONCLUSIONS

The PA83 expressed in transgenic tobacco proved to be functionally similar to PA derived from *Bacillus anthracis*. The comparable IgG levels in mice groups immunized with chloroplast derived purified PA83 and PA should eliminate the concerns regarding the protein folding and assembly. There are several reasons that make genetic engineering of the chloroplast genome the most cost effective and prudent way to produce the PA83 recombinant protein for use as a vaccine. Maternal inheritance allows for gene containment. The technology exists to harvest and store plant produced proteins making the cost of production of recombinant proteins in tobacco leaves up to 10 to 50 times cheaper than that of *E. coli* fermentation (with 20% expression levels in *E. coli*, Kusnadi *et al*., 1997).

PCR and Southern analysis confirmed the PA transgene to be present in the tobacco chloroplast genome. Immunoblot analysis confirmed the expression of 83 kDa / 63kDa PA protein. ELISA showed PA expression levels up to 14.78% total soluble protein. The *in vitro* and *in vivo* experiments prove that the PA83 is biologically functional similar to PA derived from *Bacillus antharcis*. The immunization studies showed that the chloroplast derived PA83 and PA83 derived from *Bacillus anthracis* elicited comparable immune responses and that the plant derived PA83 is biologically functionally similar to that of PA83 derived from *Bacillus antharcis*.

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